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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 4 : C12N 15/00 — A1

(11) International Publication Number: WO 89/ 01038

(43) International Publication Date: 9 February 1989 (09.02.89)

(21) International Application Number: PCT/US88/02445

(22) International Filing Date: 20 July 1988 (20.07.88)

(31) Priority Application Number: 077,188

(32) Priority Date: 24 July 1987 (24.07.87)

(33) Priority Country: US

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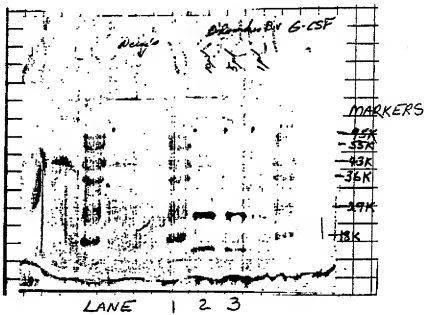
(81) Designated States: AT (European patent), AU, BE (European patent), CH (European patent), DE (European patent), DK, FI, FR (European patent), GB (European patent), IT (European patent), JP, LU (European patent), NL (European patent), NO, SE (European patent).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: PRODUCTION OF BIOLOGICALLY ACTIVE FORMS OF CSF USING A BACULOVIRUS (AcNPV)-INSECT CELL EXPRESSION SYSTEM



(57) Abstract

The expression of biologically active CSFs in insect cells (*Spodoptera frugiperda*) is disclosed. Numerous forms of CSF-1 are expressed and secreted as biologically active dimers. DNA transfer vectors and expression vectors that encode forms of CSF-1 and allow for their expression are disclosed and claimed. G-CSF is additionally expressed in insect cells in active form. DNA transfer vectors and expression vectors that encode G-CSF are disclosed and claimed.

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PRODUCTION OF BIOLOGICALLY ACTIVE FORMS OF CSF USING A BACULOVIRUS (AcNPV)-INSECT CELL EXPRESSION SYSTEM

The present invention relates to the field of molecular biology and proteins. In particular, the invention relates to the achievement of high levels of expression of biologically active colony stimulating factors using an insect cell/baculovirus expression system.

Numerous different expression systems are now available to the genetic engineer who wishes to express a cloned gene of interest. Generally the goal is the production of high levels of biologically active material. The requirements for the expression system chosen to accomplish this goal are dependent upon the nature of the protein to be expressed.

The value of utilizing prokaryotic host vector systems for the synthesis of desirable eukaryotic proteins is diminished by certain limitations inherent in such systems. For instance, the mRNA transcript or protein product of such systems may be unstable in the prokaryote. In addition, before a protein will be synthesized within a prokaryotic cell, the DNA sequence introduced into the microorganism must be free of intervening DNA sequences, nonsense sequences, and initial or terminal sequences which encode for polypeptide sequences which do not comprise the active eukaryotic protein. Further, some eukaryotic proteins require modification after synthesis (e.g., glycosylation and all membrane associated processing) to become biologically active, and prokaryotic cells are generally incapable of such modifications.

Various nonviral eukaryotic host vector systems are also available for the expression of heterologous proteins. Certain limitations are inherent in each of these systems as well. For example, high levels of expression are frequently difficult to obtain in yeast systems where autonomously replicating vectors may be unstable. Additionally, glycosylation patterns in yeast differ from those in higher eukaryotes.

Limitations encountered with mammalian host vector systems include difficulties in host cell culturing and its scale-up. The expense of mammalian cell culture media as well as a requirement for serum often restricts its use on a large scale and complicates the use of these systems for production of parenterally administered pharmaceuticals. Furthermore, levels of expression in these systems are generally substantially lower than that obtained in prokaryotic or viral expression systems.

The use of viruses in eukaryotic host-vector systems has been the subject of a considerable amount of recent investigation and speculation. However, some viral vector systems also suffer from significant disadvantages and limitations which diminish their utility. For example, a number of eukaryotic viral vectors are either tumorgenic or oncogenic in mammalian systems and create potentially health and safety problems associated with resultant gene products and accidental infection.

The baculovirus expression vector system involved in the instant invention overcomes many of the above-mentioned limitations. Baculoviruses are insect pathogenic viruses which, until recently, were studied mostly for their potential use as viral insecticides for control of agriculturally important insect pests. Because certain baculoviruses are highly virulent for pest insects, some of the most promising have been commercially developed and are used as biological pesticides (Miltenburger and Krieg 1984 Bioinsecticides:II: Baculoviridae. Adv. Biotechnol. Processes 3:291; Granados, R.R. and Federici, B.A. eds. The Biol. of Baculoviruses Vol II, Boca Raton, FL: CRC Press, Inc. 1986). Baculoviruses are very stable and are able to persist for longer times in the environment than other animal viruses. This unusual biological stability is the result of a unique association of the infectious virus particles and a viral occlusion which is a crystalline assembly of a viral encoded structural protein called polyhedrin. Late in viral replication, baculovirus particles become embedded in a protein occlusion composed of the polyhedrin protein. Insects acquire a baculovirus disease by ingesting these occluded virus (OV) which contaminate their food supply. The

polyhedrin matrix protects the virus particles in the environment and during their passage through the foregut of the insect. In the insect midgut, the alkaline pH activates the dissolution of the polyhedrin crystalline matrix resulting in the release of many viruses. The virus become absorbed by the midgut epithelial cells and initiate the infection process.

There is a second infectious form of nuclear polyhedrosis viruses (NPVs), known as the extracellular or nonoccluded virus (NOV) form, which is generated by the budding of viral nucleocapsids through the plasma membrane of the infected cells. NOVs are responsible for spreading a secondary infection via the hemolymph of the insect. It is the NOV form of the virus which is infectious in insect cell cultures; the occluded (OV) form is not infectious in cell cultures since dissolution of the crystalline matrix occurs only at high alkaline pH (i.e., pH 10.5).

The formation of NOVs and OVs occurs in a biphasic manner during the infection process. NOVs are abundantly produced before occlusion is initiated. During a typical synchronous infection of fully permissive cell lines, the majority of NOVs are produced between 12 and 24 hr post-infection (p.i.). The synthesis of polyhedrin is initiated at 20 hr p.i. and does not reach maximal levels until 48 to 72 hr p.i. The significance of this temporal regulation with respect to the expression vector system is that foreign gene products that may have adverse effects on the cell should not diminish the production of progeny NOVs to be used for further infection.

Of the 450-500 species of known baculoviruses, practically all encode for a polyhedrin protein. As previously discussed, the viral occlusion is a paracrystalline assembly of a polyhedrin monomer which, for most viruses, has an average molecular weight of 28,000-30,000 daltons (Summers, M.D. and Smith, G.E., 1978 Virology 84:390). Baculoviruses are unique among animal viruses, not only in the protective function of the viral occlusion in the viral life cycle but also because the polyhedrin gene is the most highly expressed eucaryotic virus gene known. The polyhedrin protein can accumulate to

greater than 1 mg/ml of infected cultured insect cells (70-75% of the total cellular protein) or can comprise up to 25% of the total protein of an infected insect. Although very highly expressed, neither the polyhedrin gene nor its protein is essential for viral infection or replication in cultured insect cells or insects, thus making the polyhedrin gene an ideal target for genetic manipulation.

The most extensively studied baculovirus is the Autographa californica nuclear polyhedrosis virus (AcNPV). The Autographa californica host for AcNPV is a moth commonly referred to as the alfalfa looper. Studies of the physical and functional organization of the AcNPV genome have resulted in the mapping, cloning, and sequencing of the AcNPV polyhedrin gene and its regulatory sequences (Iddekinge et al. (1983) Virology 131:561; Smith et al. (1983) J. Virol. 46:584). Not only does the polyhedrin gene exhibit a strong promoter, but expression can continue late in infection well beyond the point of repression of nearly all other baculovirus and host genes.

The genetic engineering of the baculovirus polyhedrin gene for high level expression of a heterologous protein, in this case recombinant human beta-interferon was first reported by Smith et al. Mol. Cell. Biol. 3(12):2156-2165 (1983)). Since then, human interleukin-2 has been expressed in insect cells by a baculovirus expression vector as described by Smith et al. (Proc. Natl. Acad. Sci. USA 32:8404-8408 (1985)). Recently, the synthesis of functional human T-cell leukemia virus Type I $p40^{X}$ protein using a baculovirus expression vector has been reported (Jeang, K.T., et al., J. Virol. 61:708-713 (1987)). Other heterologous proteins that have been expressed in this system are summarized in Summers et al. ("Genetic Engineering of the Genome of the Autographa californica Nuclear Polyhedrosis Virus" Banbury Report: Genetically Altered Viruses in the Environment, 22:319-329, Cold Spring Harbor Laboratory (1985)).

The baculovirus expression system has several advantages for the expression of foreign genes in comparison to other prokaryotic, yeast or mammalian cell expression vector systems. First, high levels

of expressed proteins are possible. Greater than 1.0 mg per ml of polyhedrin protein is normally produced in infected cells. Another non-essential occlusion-related viral protein, p10, is also abundantly produced and its promoter has reportedly been used to drive foreign gene expression (D.W. Miller et al. Genetic Engineering Principles & Methods 8:277-298, Setlow and Hollaender, eds. New York: Plenum Press, 1986). Using the polyhedrin gene promoter, heterologous gene expression levels never reach polyhedrin levels but are usually in the range of tens to hundreds of micrograms per ml (Summers et al. (1985), p. 321, supra). Secondly, in contrast to those produced in bacterial or yeast cells, recombinant proteins produced in insect cells may be co- and post-translationally processed in a manner similar to what occurs in mammalian cells. In at least one case, glycosylation of IFN-beta in infected insect cells has been reported (G.E. Smith et al. (1983) supra). Whereas about 40% of the natural IL-2 produced in human Jurkat cells is not glycosylated, there was no evidence of any glycosylation of the recombinant IL-2 produced in insect cells (G.E. Smith, et al., (1985), supra). In addition, correct cleavage of mammalian secretory signal peptides has been observed (G.E. Smith et al., (1983), supra; G.E. Smith et al., (1985), supra; D.W. Miller et al., (1986), supra.

As described by M. B. Ladner et al., (EMBO J. (1987) 6:2683-2698 and incorporated herein by reference, the production of biologically active CSF-1 is complicated by its high degree of posttranslational processing which includes glycosylation and dimerization. In addition there are a large number of cysteine residues, in particular, in the N-terminal portion of the protein. There are, in fact, a total of ten cysteine residues in the "long form" of CSF and seven in the "short form". Both thus contain cysteine residues at positions 7, 31, 48, 90, 102, 139, 146, the long form has additional cysteines at 157, 159 and 225. It is believed that processing to form dimer includes formation of multiple intrachain and at least one interchain disulfide bond(s). While prokaryotic systems may be employed for CSF-1, refolding protocols are necessary to produce high yields of biologically active product. It

would be desirable to have a high level expression system that produces biologically active dimers of CSF-1.

As described in PCT Publication No. WO88/01297 published February 25, 1988, and incorporated herein by reference, the native DNA sequence encoding G-CSF has not proved to be effective for the production of high levels of G-CSF in prokaryotic hosts. Not only does the insect cell expression system provide an intriguing alternative but the possibilities of achieving the appropriate post-translational processing and recovery of G-CSF from the culture medium would be advantageous in the production of pharmaceutically useful CSFs.

The invention relates, in one respect, to methods for producing by recombinant DNA technology biologically active CSFs and in the case of CSF-1, a dimerized form of CSF-1, using host insect cells infected with a recombinant baculovirus expression vector. Accordingly, one aspect of the invention relates to growing the infected insect cells under suitable conditions to produce the desired recombinant CSFs and recovering the biologically active polypeptide or dimerized polypeptide from the culture medium.

In another aspect, the invention is directed to recombinant baculovirus expression vectors which are capable of effecting the expression of CSFs, to the host cells infected with such vectors, and to cultures thereof.

One aspect of the invention concerns recombinant baculovirus expression vectors in which CSFs are expressed under the transcriptional control of a baculovirus promoter. In one aspect of the invention the baculovirus promoter is the polyhedrin gene promoter.

Yet another aspect of the invention relates to recombinant baculovirus expression vectors that are capable of expressing CSFs and which may encode either the long or short forms of CSF-1, muteins thereof, CSF-1 in which the amino terminal 4 codons are altered, or G-CSF.

Also, aspects of the invention are the recombinant baculovirus transfer vectors which are used to transfer the desired recombinant gene into the baculovirus genome.

Figure 1 shows the DNA sequence of the CSF-1 gene from pcCSF-17.

Figure 2 shows the DNA sequence of original cDNA clone number 4. The sequence is missing a few nucleotides at the 5' end compared to the LCSF sequence in pcDBhuCSF-4 which was completed by substitution of upstream codons from pcCSF-17 (see Example 2, Section A.2.)

Figure 3 shows the DNA sequence of G-CSF from pP12.

Figure 4 shows the DNA sequence comparison between the recombinant baculovirus transfer vectors pAcC1-C5. The carrots indicate restriction endonuclease cleavage sites.

Figure 5 shows the DNA sequence comparison for the regions around the CSF-1 translational start codon between the recombinant baculovirus transfer vectors pAcM1, pAcM2, pAcM3, pAcM4, pAcM6, pAcM10 and the polyhedrin gene.

Figure 6 shows SDS-PAGE analysis of immunoprecipitated baculovirus expression system products.

Figure 7 shows Western blot analysis of G-CSF expressed by the baculovirus system.

Figure 8 shows expression levels of various recombinant polypeptides in the baculovirus expression vector system.

Modes for Carrying Out the Invention

A. Definitions

"Colony stimulating factor-1 (CSF-1)" refers to a protein which exhibits the spectrum of activity understood in the art for CSF-1, i.e., when applied to the standard *in vitro* colony stimulating assay of Metcalf, D., *J. Cell Physiol.* (1970) 76:89, it results in the

formation of primarily macrophage colonies. Native CSF-1 is a glycosylated dimer; dimerization may be necessary for activity. Contemplated within the scope of the invention and within the definition of CSF-1 are both the dimeric and monomeric forms. The monomeric form may be converted to the dimeric form by in vitro provision of host intracellular conditions suitable for dimerization of the monomer.

There appears to be some species specificity: Human CSF-1 is operative both on human and on murine bone marrow cells; murine CSF-1 does not show activity with human cells. Therefore, "human" CSF-1 should be positive in the specific murine radioreceptor assay of Das, S. K., et al., Blood (1981) 58:630, although there is not necessarily a complete correlation. The biological activity of the protein will generally also be inhibited by neutralizing antiserum to human urinary CSF-1 (Das, S. K., et al., supra). However, in certain special circumstances (such as, for example, where a particular antibody preparation recognizes a CSF-1 epitope not essential for biological function, and which epitope is not present in the particular CSF-1 mutein being tested) this criterion may not be met.

Certain other properties of CSF-1 have been recognized more recently, including the ability of this protein to stimulate the secretion of series E prostaglandins, interleukin-1, and interferon from mature macrophages (Moore, R., et al., Science (1984) 223:178; Ralph, P. et al. (1986) Immunobiol., 172:194; Ralph, P. et al. (1987) Cell. Immunol., 105:270). The mechanism for these latter activities is not at present understood, and for purposes of definition herein, the criterion for fulfillment of the definition resides in the ability to stimulate the formation of monocyte/macrophage colonies using bone marrow cells from the appropriate species as starting materials, under most circumstances (see above) the inhibition of this activity by neutralizing antiserum against purified human urinary CSF-1, and, where appropriate for species type, a positive response to the radioreceptor assay. (It is known that the proliferative effect of CSF-1 is restricted to cells of mononuclear phagocytic lineage (Stanley, E. R., The Lymphokines (1981), Stewart, W. E., II, et al.,

ed., Humana Press, Clifton, JN), pp. 102-132) and that receptors for CSF-1 are restricted to these cell types (Byrne, P. V., et al., Cell Biol. (1981) 91:848)).

Figure 1 shows the amino acid sequence for a particular form of human CSF-1 encoded by the recombinant cDNA clone pcCSF-17 (Kawasaki, E.S., et al. (1985), Science 230:291). This protein contains 224 amino acids in the mature sequence and a leader sequence of 32 amino acids. The protein produced as the expression product of this clone is active in assays specific for CSF-1, namely, the bone marrow proliferation assay (wherein the activity is destroyed by addition of anti-CSF-1 antibodies), colony stimulation assays, and a radioreceptor assay. Further characterization of the biological and molecular properties of human and murine csF-1 is disclosed by P. Ralph et al. in Molecular Basis of Lymphokine Action (D. R. Webb et al., eds., The HUMANA Press, Inc., 1987, pp. 295-311).

For convenience, the mature protein amino acid sequence of the monomer portion of a dimeric protein shown in Figure 1, deduced from the cDNA clone illustrated herein, is designated mCSF-1 (mature CSF-1). Figure 1 shows the presence of a 32 residue putative signal sequence, which is presumably cleaved upon secretion from mammalian cells; mCSF-1 is represented by amino acids 1-224 shown in that figure. Specifically included in the definition of human CSF-1 are muteins which monomers and dimers are mCSF-1 and related forms of mCSF-1. CSF-1 derived from other species may fit the definition of "human" CSF-1 by virtue of its display of the requisite pattern of activity as set forth above with regard to human substrate.

Also for convenience, the amino acid sequence of mCSF-1 will be used as a reference and other sequences which are substantially equivalent to this in terms of CSF-1 activity will be designated by referring to the sequence shown in Figure 1. The substitution of a particular amino acid will be noted by reference to the amino acid residue which it replaces. Thus, for example, ser_{90}CSF-1 refers to the protein which has the sequence shown in Figure 1 except that the amino acid at position 90 is serine rather than cysteine. Numerous

muteins have been described in commonly owned PCT Publication Nos. WO86/04607 published August 14, 1986 and PCT Publication No. WO88/03173 published May 5, 1988 each incorporated herein by reference and are included within the definition of CSF-1 according to the invention so long as the biological activity of CSF-1 is maintained. It is expected, of course, that such modifications may quantitatively or qualitatively affect the activity, either by enhancing or diminishing the activity of the protein in various assays.

Additional CSF-1 proteins, which have the same colony stimulating activity described in Metcalf supra, contain substantially longer amino acid sequences deduced from the recovered cDNA, which include a 298 amino acid "extra" segment at approximately residue 150 of the pcCSF-17 encoded protein and expression of this cDNA results in CSF-1 activity in these specific CSF-1 assays, as well.

For convenience, the CSF-1 encoded by pcCSF-17 will be referred to as the "short form" of the protein, and for consistency with previous designations, as "mature" or mCSF-1. The 522 amino acid sequence encoded in, for example, pcDBhuCSF-4 and its corresponding clones will be referred to as the "long form", or LCSF-1. This sequence is shown in Figure 2. LCSF-1 is further described in PCT Publication No. WO87/06954 published November 19, 1987, and in commonly owned PCT Publication No. WO88/03173 published May 5, 1988. The abbreviations used herein also include "huCSF-1" for all human forms of the protein.

"G-CSF" as used herein means a protein having the effect of stimulating the production of primarily granulocyte colonies or granulocyte-macrophage colonies in a colony forming assay using bone marrow cell progenitors of an appropriate species. A protein having this activity has the deduced amino acid sequence shown in Figure 3 as disclosed in commonly owned EP Publication No. 256,843 published February 24, 1988, and in PCT Publication No. WO88/01297 published February 25, 1988 incorporated herein by reference.

G-CSF may be isolated from the MIA PaCa-2 cell line. In addition, G-CSF specific RNA sequences are clearly detectable in the mRNA of LD-1 cells and 5637 cells. Thus, G-CSF obtained by cloning

mRNA of LD-1 cells and 5637 cells. Thus, G-CSF obtained by cloning from these cell lines as well as any other cell line which is G-CSF probe positive, is considered within the scope of the invention. Mature G-CSF produced by the LD-1 cell line has been isolated and amino-acid sequenced. 65% of the LD-1 produced protein has a Thr residue at the NH_2-terminus and 35% has a Pro residue at the NH_2-terminus. Further disclosure on the cloning and expression of G-CSF are found in commonly owned PCT Publication No. WO88/01297 published February 25, 1988 and EP Publication No. 256,843 (supra).

The mature G-CSF protein amino acid sequence shown in Figure 3, deduced from the cDNA clone, is designated mG-CSF (mature G-CSF) beginning at the amino acid residue threonine designated +1. There is a 30 residue putative signal sequence, which is presumably cleaved upon secretion from mammalian cells; mG-CSF is represented by amino acids 1-174 shown in that figure. Specifically included in the definition of human G-CSF are muteins which monomers and dimers, if any, are G-CSF and related forms of G-CSF, designated by their differences from mG-CSF. G-CSF derived from other species may fit the definition of "human" G-CSF by virtue of its display of the requisite pattern of activity as set forth above with regard to human substrate.

Also for convenience, the amino acid sequence of G-CSF will be used as a reference and other sequences which are substantially equivalent to this in terms of G-CSF activity will be designated by referring to the sequence shown in Figure 3. The substitution of a particular amino acid will be noted by reference to the number of the amino acid residue which it replaced. Thus, for example, ser_{60} G-CSF refers to the protein which has the sequence shown except that the amino acid at position 60 is serine rather than proline.

As is the case for all proteins, the precise chemical structure of CSF-1 or G-CSF depends on a number of factors. As ionizable amino and carboxyl groups are present in the molecule, a particular protein may be obtained as an acidic or basic salt, or in neutral form. All such preparations which retain their activity when placed in suitable environmental conditions are included in the definition. Further, the primary amino acid sequence may be augmented

by derivatization using sugar moieties (glycosylation) or by other supplementary molecules such as lipids, phosphate, acetyl groups and the like, more commonly by conjugation with saccharides. The primary amino acid structure may also aggregate to form complexes. Certain aspects of such augmentation are accomplished through post-translational processing systems of the producing host; other such modification may be introduced in vitro. In any event, such modifications are included in the definition so long as the activity of the protein, as defined above, is not destroyed. It is expected, of course, that such modifications may quantitatively or qualitatively affect the activity, either by enhancing or diminishing the activity of the protein in the various assays. Further, individual amino acid residues in the chain may be modified by oxidation, reduction, or other derivatization, and the protein may be cleaved to obtain fragments which retain activity. Such alterations which do not destroy activity do not remove the protein sequence from the definition.

Modifications to the primary structure itself by deletion, addition, or alteration of the amino acids incorporated into the sequence during translation can be made without destroying the activity of the protein. Such substitutions or other alterations result in proteins having an amino acid sequence which falls within the definition of proteins "having an amino acid sequence substantially equivalent to that of CSF-1 or G-CSF."

"Operably linked" refers to juxtaposition such that the normal function of the components can be performed. Thus, a coding sequence "operably linked" to control sequences refers to a configuration wherein the coding sequence can be expressed under the control of these sequences.

"Control sequences" refers to DNA sequences necessary for the expression of an operably linked coding sequence in a particular host organism. Eukaryotic cells including the insect cells of the instant invention are known to utilize promoters and polyadenylation signals.

"Expression system" refers to DNA sequences containing a desired coding sequence and control sequences in operable linkage, so that hosts transformed with these sequences are capable of producing the encoded proteins. These DNA sequences may also direct the synthesis of the encoded proteins in an in vitro cellular environment. In order to effect transformation, the expression system may be included on a transfer vector; however, the relevant DNA may then also be integrated into the viral chromosome to result in a recombinant viral genome.

As used herein "cell", "cell line", and "cell culture" are used interchangeably and all such designations include progeny. Thus "transformants" or "transformed cells" includes the primary subject cell and cultures derived therefrom without regard for the number of transfers. It is also understood that all progeny may not be precisely identical in DNA content, due to deliberate or inadvertent mutations. Mutant progeny which have the same functionality as screened for in the originally transformed cell are included. Where distinct designations are intended, it will be clear from the context.

"Infection" as used herein refers to the invasion of cells by pathogenic viral agents where conditions are favorable for their replication and growth. "Transfection" refers to a technique for infecting cells with purified nucleic acids by adding calcium chloride to solutions of DNA containing phosphate or other appropriate agents such as dextran sulfate thereby causing the DNA to precipitate and be taken up into the cells.

"Recombinant transfer vector" refers to a plasmid containing a "heterologous" gene under the control of a functional promoter (e.g., polyhedrin or p10 promoter) and flanked by viral sequences. The "recombinant expression vector" is formed after cotransfection of the recombinant transfer vector and wild-type baculovirus DNA into host insect cells whereupon homologous recombination occurs between the viral sequences flanking the heterologous gene and the homologous sequences in the wild-type viral DNA. This results in the replacement of wild-type sequences in the virus with the transfer vector sequences

between the crossover points. The recombinant expression vector is the recombinant viral DNA containing the desired heterologous gene.

B. General Description

Interest in the growth and differentiation of granulocytes and macrophages from bone marrow progenitors has prompted the study of the colony-stimulating factors (CSFs) that regulate these processes. The subject has been recently reviewed by D. Metcalf in Blood, 67:257 (1986). One of these factors, termed macrophage-CSF (M-CSF or CSF-1) stimulates the in vitro growth of predominantly macrophage colonies from bone marrow stem cells. Another of these factors, termed granulocyte-CSF (G-CSF) stimulates the in vitro growth of predominantly granulocyte colonies from bone marrow stem cells.

The CSF-1 proteins of the invention are capable both of stimulating monocyte-precursor-macrophage cell production from progenitor marrow cells, thus enhancing the effectiveness of the immune system, and of stimulating such functions of these differentiated cells as the secretion of lymphokines in the mature macrophages.

In one application, these proteins are useful as adjuncts to chemotherapy. It is well understood that chemotherapeutic treatment results in suppression of the immune system. Often, although successful in destroying the tumor cells against which they are directed, chemotherapeutic treatments result in the death of the subject due to this side effect of the chemotoxic agents on the cells of the immune system. Administration of CSF-1 to such patients, because of the ability of CSF-1 to mediate and enchance the growth and differentiation of bone marrow-derived precursors into macrophages, results in a restimulation of the immune system to prevent this side effect, and thus to prevent the propensity of the patient to succumb to secondary infection. Other patients who would be helped by such treatment include those being treated for leukemia through bone marrow transplants; they are often in an immunosuppressed state to prevent rejection. For these patients also, the immunosuppression could be reversed by administration of CSF-1.

In addition to the cDNA encoding the 224 amino acid CSF-1 monomer described above, cDNAs from both human and murine systems have been obtained which include DNA encoding an additional 298 amino acid sequence. These cDNAs express to produce "long form" CSF-1 proteins which fulfill the criteria for biological activity of this protein.

In general outline, the human cDNA encoding the 224 amino acid form of the protein was used as a probe to recover the sequences encoding murine CSF-1 from a cDNA bank prepared in lambda gt10 from L-929 mRNA which had been enriched for CSF-1 production capability. Two clones were recovered which encode similar 520 amino acid proteins. The clones diverge dramatically in the 3' untranslated region. One of the 3' untranslated regions is more than 2 kb and bears little resemblance to the corresponding human sequences; the other, shorter clone contains approximately 500 bp in the untranslated region and shows considerable homology to the corresponding human DNA.

The longer forms of CSF-1 obtained from the murine library were then used as a basis to prepare probes to retrieve longer human sequences, if any. Based on comparison of the murine cDNAs to the human genomic sequence, a region of the gene previously thought to lie in an intron region putatively encodes an amino acid sequence showing considerable homology to the "extra" 298 amino acid segment contained in the murine sequence. This permitted construction of an oligonucleotide probe based on the "extra" DNA which had been, in the murine system, translated to protein. However, since the human genomic sequence was available, the probe was designed to accomodate the precise human sequence.

While pcCSF-17 had been prepared from MIA PaCa mRNA enriched for CSF-1-encoding materials, a subsequent cDNA library was prepared from total mRNA extracted from MIA PaCa cells and cloned into gt10. The gt10 library was first screened using pcCSF-17 sequences as probe, and selected probe-positive candidates were screened using an oligonucleotide probe based on the "extra" translated sequence of the murine cDNA, but modified to correspond to the related region in the human genome. Several clones encoding a corresponding "long form" of

a human protein were also obtained. Of course, the availability of DNA encoding each of these sequences provides the opportunity to modify the codon sequence so as to generate mutein forms also having CSF-1 activity.

For example, glycosylation sites within the coding sequence may be predicted by the presence of the sequence Asn. x. Ser or Thr which is found at glycosylated Asn residues in all known glycoproteins (Hughes, R.C. (1983) Glycoproteins New York: Chapman and Hall, p. 42). The Asn residue within this sequence, for example, may be substituted by site specific mutagenesis to remove the glycosylation site. As disclosed herein, two such substitutions were carried out in the CSF-1 molecule.

C. Methods Employed

C.1. Transformations and Transfections

Transformation of E. coli cells was done according to procedures set forth in T. Maniatis, E.F. Fritsch and J. Sambrook Molecular Cloning: A Laboratory Manual (1982) Cold Spring Harbor Press.

Transfections of Sf9 cells are accomplished using a modification of the calcium phosphate precipitation technique (Graham, F.L. et al., 1973, Virology 52:456) as modified for insect cells (Burand, J.P. et al. (1980), Virol., 101:286; Carstens, E.B. et al. (1980) Virol., 101:311) and further described by Summers, M.D. and Smith, G.E. (A Manual of Methods for Baculovirus Vectors and Insect Cell Culture Procedures, Texas A & M Press: 1986)

C.2. Probing mRNA by Northern Blot; Probe of cDNA or Genomic Libraries

RNA is fractionated for Northern blot by agarose slab gel electrophoresis under fully denaturing conditions using formaldehyde (Maniatis, T., et al., supra pp 202-203) or 10 mM methyl mercury (CH_3HgOH) (Bailey, J. M., et al., Anal. Biochem. (1976) 70:75-85; and Sehgal, P. B., et al., Nature (1980) 288:95-97) as the denaturant.

For methyl mercury gels, 1.5% gels are prepared by melting agarose in running buffer (100 mM boric acid, 6 mM sodium borate, 10 mM sodium sulfate, 1 mM EDTA, pH 8.2), cooling to 60°C and adding 1/100 volume of 1 M CH_3HgOH. The RNA is dissolved in 0.5 x running buffer and denatured by incubation in 10 mM methyl mercury for 10 minutes at room temperature. Glycerol (20%) and bromophenol blue (0.05%) are added for loading the samples. Samples are electrophoresed for 500-600 volt-hr with recirculation of the buffer. After electrophoresis, the gel is washed for 40 minutes in 10 mM 2-mercaptoethanol to detoxify the methyl mercury, and Northern blots prepared by transferring the RNA from the gel to a membrane filter.

cDNA or genomic libraries are screened using the colony or plaque hybridization procedure. Bacterial colonies, or the plaques for phage are lifted onto duplicate nitrocellulose filter papers (S & S type BA-85). The plaques or colonies are lysed and DNA is fixed to the filter by sequential treatment for five minutes with 500 mM NaOH, 1.5 M NaCl. The filters are washed twice for five minutes each time with 5 x standard saline citrate (SSC) and are air dried and baked at 80°C for two hours.

The gels for Northern blot or the duplicate filters for cDNA or genomic screening are prehybridized at 25-42°C for 6-8 hours with 10 ml per filter of DNA hybridization buffer without probe (0-50% formamide, 5-6 x SSC, pH 7.0, 5 x Denhardt's solution (polyvinylpyrrolidine, plus Ficoll and bovine serum albumin; 1 x = 0.02% of each), 20-50 mM sodium phosphate buffer at pH 7.0, 0.2% SDS, 20 μg/ml poly U (when probing cDNA), and 50 μg/ml denatured salmon sperm DNA). The samples are then hybridized by incubation at the appropriate temperature for about 24-36 hours using the hybridization buffer containing kinased probe (for oligomers). Longer cDNA or genomic fragment probes were labeled by nick translation or by primer extension.

The conditions of both prehybridization and hybridization depend on the stringency desired, and vary, for example, with probe length. For example, conditions for relatively long (e.g., more than

30-50 nucleotide) probes may employ a temperature of 42°C-55°C and hybridization buffer containing about 20%-50% formamide. For the lower stringencies needed for oligomeric probes of about 15 nucleotides, lower temperatures of about 25°C-42°C, and lower formamide concentrations (0%-20%) are employed. For longer probes, the filters may be washed, for example, four times for 30 minutes, each time at 40°C-55°C with 2 x SSC, 0.2% SDS and 50 mM sodium phosphate buffer at pH 7, then washed twice with 0.2 x SSC and 0.2% SDS, air dried, and are autoradiographed at -70°C for 2 to 3 days. Washing conditions are somewhat less harsh for shorter probes. Other conditions are known in the art that may provide the results disclosed herein.

C.3. Vector Construction

Construction of suitable vectors containing the desired coding and control sequences employs standard ligation and restriction techniques which are well understood in the art and are described in Maniatis, T. et al., supra. Isolated plasmids, DNA sequences, or synthesized oligonucleotides are cleaved, tailored, and religated in the form desired.

Site specific DNA cleavage is performed by treating with the suitable restriction enzyme (or enzymes) under conditions which are generally understood in the art, and the particulars of which are specified by the manufacturer of these commercially available restriction enzymes. See, e.g., New England Biolabs, Product Catalog. In general, about 1 μg of plasmid or DNA sequence is cleaved by one unit of enzyme in about 20 μl of buffer solution; in the examples herein, typically, an excess of restriction enzyme is used to insure complete digestion of the DNA substrate. Incubation times of about one hour to two hours at about 37°C are workable, although variations can be tolerated. After each incubation, protein is removed by extraction with phenol/chloroform, and may be followed by ether extraction, and the nucleic acid recovered from aqueous fractions by precipitation with ethanol. If desired, size separation of the cleaved fragments may be performed by polyacrylamide gel or

agarose gel electrophoresis using standard techniques. A general description of size separations is found in Methods in Enzymology (1980) 65:499-560.

Restriction cleaved fragments may be blunt ended by treating with the large fragment of E. coli DNA polymerase I (Klenow) in the presence of the four deoxynucleotide triphosphates (dNTPs) using incubation times of about 15 to 25 minutes at 20 to 25°C in 50 mM Tris pH 7.6, 50 mM NaCl, 6 mM $MgCl_2$, 6 mM dTT and 5-10 μM dNTPs. The Klenow fragment fills in at 5' sticky ends but chews back protruding 3' single strands, even though the four dNTPs are present. If desired, selective repair can be performed by supplying only one of the, or selected, dNTPs within the limitations dictated by the nature of the sticky ends. After treatment with Klenow, the mixture is extracted with phenol/chloroform and ethanol precipitated. Treatment under appropriate conditions with S1 nuclease results in hydrolysis of any single-stranded portion.

Synthetic oligonucleotides may be prepared by the triester method of Matteucci, et al., J. Am. Chem. Soc. (1981) 103:3185-3191 or using automated synthesis methods. Kinasing of single strands prior to annealing or for labeling is achieved using an excess, e.g., approximately 10 units of polynucleotide kinase to 1 nM substrate in the presence of 50 mM Tris, pH 7.6, 10 mM $MgCl_2$, 5 mM dithiothreitol, 1-2 mM ATP. If kinasing is for labeling or probe, the ATP will contain high specific activity gamma ^{32}P.

Ligations are performed in 15-30 μl volumes under the following standard conditions and temperature: 20 mM Tris-Cl pH 7.5, 10 mM $MgCl_2$, 10 mM dTT, 33 μg/ml BSA, 10 mM-50 mM NaCl, and either 40 μM ATP, 0.01-0.02 (Weiss) units T4 DNA ligase at 0°C (for "sticky end" ligation) or 1 mM ATP, 0.3-0.6 (Weiss) units T4 DNA ligase at 14°C (for "blunt end" ligation). Intermolecular "sticky end" ligations are usually performed at 33-100 μg/ml total DNA concentrations (5-100 nM total end concentration). Intermolecular blunt end ligations (usually employing a 10-30 fold molar excess of linkers) are performed at 1 μM total ends concentration.

In vector construction employing "vector fragments", the vector fragment is commonly treated with bacterial alkaline phosphatase (BAP) in order to remove the 5' phosphate and prevent religation of the vector. BAP digestions are conducted at pH 8 in approximately 150 mM Tris, in the presence of Na^+ and Mg^{+2} using about 1 unit of BAP per μg of vector at 60°C for about one hour. In order to recover the nucleic acid fragments, the preparation is extracted with phenol/chloroform and ethanol precipitated. Alternatively, religation can be prevented in vectors which have been double digested by additional restriction enzyme digestion of the unwanted fragments.

C.4. Modification of DNA Sequences

For portions of vectors derived from cDNA or genomic DNA which require sequence modifications, site specific primer directed mutagenesis is used. This technique is now standard in the art, and is conducted using a primer synthetic oligonucleotide complementary to a single stranded phage DNA to be mutagenized except for limited mismatching, representing the desired mutation. Briefly, the synthetic oligonucleotide is used as a primer to direct synthesis of a strand complementary to the phage, and the resulting double-stranded DNA is transformed into a phage-supporting host bacterium. Cultures of the transformed bacteria are plated in top agar, permitting plaque formation from single cells which harbor the phage.

Theoretically, 50% of the new plaques will contain the phage having, as a single strand, the mutated form; 50% will have the original sequence. The plaques are hybridized with kinased synthetic primer at a temperature which permits hybridization of an exact match, but at which the mismatches with the orignal strand are sufficient to prevent hybridization. Plaques which hybridize with the probe are then picked, cultured, and the DNA recovered. Details of site specific mutation procedures are described below in specific examples.

C.5. Verification of Construction

In the constructions set forth below, correct ligations for plasmid construction are confirmed by first transforming E. coli strain MM294, or other suitable host with the ligation mixture. Miniprep DNA was prepared according to Ish-Horowicz, D. et al. (Nucl. Acids Res. (1981) 9:2989) and screened by restriction analysis. DNA may be further analyzed by restriction and/or sequenced by the dideoxy method of Sanger, F., et al., Proc. Natl. Acad. Sci. (USA) (1977) 74:5463 as further described by Messing, et al., Nucleic Acids Res. (1981) 9:309, or by the method of Maxam, et al., Methods in Enzymology (1980) 65:499.

C.6. Transfer Vector Construction

Because the genome of AcNPV is so large (125kb), there are too many restriction sites to allow site-specific insertion of heterologous genes. Therefore, it is necessary to derive recombinant virus, containing the gene to be expressed, through homologous recombination between viral DNA and genetically engineered chimeric plasmids called transfer vectors.

The transfer vectors which have been described by Smith, G.E., et al., 1983, supra, were originally constructed by cloning the AcNPV EcoRI-1 fragment containing the polyhedrin gene into the EcoRI site of E. coli plasmid pUC8 (Vieira, J., et al., Gene 19:259-268 (1982)). A series of plasmids or transfer vectors having single BamHI cloning sites at various locations in the polyhedrin gene were created as described (Smith et al., 1983, supra). One of these, pAc373, has a single BamHI site 50 bp downstream from the polyhedrin cap site i.e., 8 bp before the polyhedrin ATG translation initiation codon (Smith et al., (1985), supra and in U.S. Patent No. 4,745,051). The transfer vectors, pAc610 and pAc611 have the polylinker from M13mp10 and M13mp11, respectively, inserted at this BamHI site of pAc373 (Luckow, V.A. et al. (1988) Biotechnol. 6:47-55. Partial nucleotide sequence of pAc401 and pAc436 transfer vectors for the production of polyhedrin/foreign gene fusion proteins has been reported (Summers, M.D. et al., 1987, p. 53, supra).

C.7. Preparation and Isolation of Recombinant AcNPV Virus

Detailed methods for the generation of recombinant virus can be found in European Patent Application No. 0127839 to G.E. Smith and M.D. Summers of the Texas A & M University System. In general, 2 μg of genetically engineered transfer vector DNA and 1 μg of AcNPV viral DNA are cotransfected onto monolayer culture cells of Spodoptera frugiperda. The infected cells usually show viral occlusions by day 3 or 4, with 10-50% of the cells being infected. The virus titer of the medium is expected to be 10^7 pfu/ml and 0.1%-0.5% are expected to be recombinant virus.

Several methods for the detection of recombinant virus are known in the art. Visual detection of the plaques is best achieved using a low power dissecting microscope and observing the plaques on inverted plates with a black background and illumination from the side. More unequivocal methods for detecting recombinants are plaque hybridization using DNA probes to the cloned gene. Antibody probes to the product of the cloned gene may also be employed.

Isolation of the recombinant virus is achieved through plaque purification of serially infected monolayer cells overlayed with soft agar. After two or three cycles the recombinant virus would be seen as separate plaques showing the characteristic occlusion-negative morphology. The plaques, containing about 10,000 pfu of virus, are picked using a sterile Pasteur pipet and transferred to 2 ml of medium.

C.8. Electrophoretic Analysis of Expression Products

In order to concentrate expression products, culture supernatants were incubated with Protein A-Sepharose CL-4B beads cross-linked to antibody to either CSF-1 or G-CSF for 1 hour. The beads were then pelleted and the supernatants reserved. The beads were washed once in .5 ml buffer containing 1M LiCl, 20 mM Tris-HCl pH 8.0 and 0.5% NP40, three times in .5 ml buffer containing 150 mM NaCl, 20 mM Tris-HCl pH8 and 0.5% NP40 and then suspended in sample loading buffer lacking reducing agent. The samples were then heated to 37°C

for 5 minutes, pelleted and the supernatant removed to new tubes These supernatants were adjusted to contain 0.14 M beta-mercaptoethanol or 20 mM DTT, heated to 100°C for 4 min. and run on SDS-PAGE. SDS-PAGE was performed essentially according to the procedure of Laemmli (Nature (1970) 227:680-685).

Immunoblotting procedures have been described in commonly owned EP Publication No. 219,286 published April 22, 1987 essentially as described below. Immunoblotting of the gel onto nitrocellulose (Schleicher and Schuell, 0.45 μmeter) was performed in a Bio-rad Trans-blot cell at 35 V for one hour at room temperature essentially according to published methods (Towbin et al. (1979) Proc. Natl. Acad. of Sci., USA, 76:4350-4354; Bittner et al. (1980) Anal. Biochem., 102:459-0471; Burnette et al. (1981) Anal. Biochem., 112:195-203). Following transfer, nonspecific antibody binding sites on the nitrocellulose were blocked by incubation for 30 minutes at room temperature with gentle agitation in 250 ml of 0.1% Tween 20 in phosphate buffered saline (PBS). Then the blot was washed three times with gentle agitation at room temperature for five minutes each in 250 ml volumes of 0.1% nonfat dry milk, 0.1% ovalbumin in PBS, and incubated with gentle agitation for three hours at room temperature in 5 ml of a 1/500 to 1/1000 dilution in the preceding buffer of rabbit antiserum against G-CSF (supplied by BABCO). After washing three times as described above, the blot was incubated for one hour at room temperature with 5 ml of a 1/2000 dilution of goat anti-rabbit IgG conjugated to horseradish peroxidase (this conjugate supplied by Zymed) and washed again three times as above.

In order to concentrate expression products, culture supernatants were incubated with Protein A-Sepharose CL-4B beads cross-linked to antibody to a CSF protein for 1 hour. The beads were then pelleted and the supernatants reserved. The beads were washed once in .5 ml buffer containing 1M LiCl, 20 mM Tris-HCl pH 8.0 and 0.5% NP40, three times in .5 ml buffer containing 150 mM NaCl, 20 mM Tris-HCl pH8 and 0.5% NP40 and then suspended in sample loading buffer lacking reducing agent. The samples were then heated to 37°C for 5 minutes, pelleted and the supernatant removed to new tubes These

supernatants were adjusted to contain 0.14 M beta-mercaptoethanol or 20 mM DTT, heated to 100°C for 4 min. and run on SDS-PAGE.

C.9. Insect Cell Culture

Methods for insect cell cultures are well known in the art and detailed procedures for their cultivation can be found in Summers, M.D. et al. (1987, supra), EP Publication No. 127,839 to G.E. Smith et al. or in U.S. Patent No. 4,745,051 (supra). The insect expression host of the current invention, Spodoptera frugiperda (Sf9) is well suited to the production of heterologous proteins because of its ability to grow in either monolayer or suspension culture.

As monolayer cultures, Spodoptera frugiperda cells will divide every 18-24 hours depending on the culture media. The cells do not require carbon dioxide to maintain the pH of the medium and they will grow well at temperatures between 25-30°C. Subculturing is done 2 or 3 times a week when the cells are confluent. Because insect cells are loosely adherent they are easily resuspended without the need of proteases.

Suspension culture conditions will vary depending on the medium and culture volume and should be determined empirically. Subculturing is required when the cell density reaches $2x10^6$ cells/ml by replacing 80% or more of the culture with an equal volume of fresh medium. With suspension cultures larger than 500 ml it becomes necessary to aerate by either bubbling or diffusion.

Preferred media and culture conditions have been described by Inlow, D. et al. (1987) "Large-scale insect culture for recombinant protein production, "Presented at Symposium on Strategies in Cell-Culture Scale-Up at the American Chemical Society National Meeting in New Orleans, Louisiana. Appropriate serum-free media include:

(a) a basal medium;

(b) a lipid component; and

(c) a peptone component.

A "basal medium" is herein defined as a nutrient mixture of inorganic salts, sugars, amino acids, optionally also containing vitamins, organic acids and/or buffers. Basal media together with supplements provide the nutrients necdessary to support cell life, growth and reproduction. The preferred basal media used as the starting point for the preparation of the serum free media of the present invention contain neither serum, nor proteins, nor preferably any peptones. The choice of basal medium for the preparation of the media of this invention is not critical. The basal medium can also be considered optinal in the sense that appropriate peptone and lipid components can be selected which provide such necessary nutrients as amino acids and vitamins required to support cell life, growth and reproduction.

As indicated, IPL-41 is a preferred basal medium for the preparation of the media used in this invention. IPL-41 basal medium is commercially available from a number of vendors and is described in Weiss, et al. In Vitro, 17(6):495-502 (June 1981) and in Weiss et al., CRC Press, supra, pp. 70-72 (1986). Table 1 of Weiss et al. (In Vitro at page 496, and Table 3 of Weiss et al. CRC Press, at pages 71-72 outline all the components of IPL-41 and provide their proportions in mg/l; said tabless are herein incorporated by reference. At page 497 of Weiss et al. (In Vitro), the preparation of the complete medium IPL-41 is described wherein tryptose phosphate broth (TPB) and fetal bovine serum (FBS) are added. The IPL-41 basal medium employed in preparing the serum-free media of this invention does not contain tryptose phosphate broth (TPB) or fetal bovin serum (FBS).

The serum-free media of this invention can further comprise a protective agent. Such a protective agent is a preferred component of the media of this invention, especially under well-aerated culture conditions. Therefore, the serum-free media of this invention include:

(a) a basal medium;

(b) a lipid component;

(c) a peptone component; and

(d) a protective agent.

A protective agent is necessary to prevent cell damage and death under well-aerated conditions as found in well agitated and sparged cultures. The protective agent prevents a disintegration/clumpding phenomenon of insect cells growth in shake flasks and adherence of the cells to the vessel walls. Further, the protective agent reduces the amont of cellular debris in shake flask cultures indicating that cell lysis is reduced by the presence of the protectant. The protective agent further preferably acts as an anti-foaming agent preventing the loss of cells from the free suspension into a foam layer, and can act as a bubble surface tension reducing agent and/or as a cell surface stabilizing agent and/or as a vciscosifying agent.

Protective agents are herein defined as non-toxic, water soluble compounds that functionally act to protect insect cells from damage and death in agitated and sparged insect cell culture. The protective agents of this invention are preferably non-toxic, water soluble polymers. A protective agent candidate can be selected by first confirming that it is not toxic to the insect cells to be cultured by methods known to those skilled in the art of insect cell culture, for example, by adding it to a suspension or monolayer of the insect cells of choice for cultivation and comparing the growth of the culture to a control. Then, the non-toxic protective agent candidates can be tested for protective ability by adding the candidate agent to an agiated or sparged culture of the insect cells of choice at small scale and observing viability and growth rate of the cells of said culture to the viability and growth rate of the cells in a control culture.

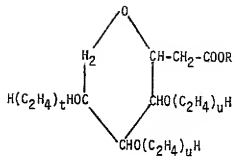
The protective agents in the media of this invention are preferably cell surface stabilizing agents and/or viscosifying agents and/or bubble surface tension reducing agents. Examples of preferred protective agents are hdydroxyethyl starch, methyl cellulose, carboxymethyl cellulosed (as, sodium carboxydmethyl cellulose),

dextrant sulfate, piolyvinylpyrrolidone, ficoll, alginic acid, polypropyleneglycol, and non-toxic polymeric detergents. Preferred non-toxic, non-ionic polymeric detergents are block polymers of propylene oxide and ethylene oxide (polyoxypropylene polyoxyethylene condensates), preferably Pluronic polyols, such as, Pluronic F68, F77, F88 and F108, preferably F68 and F88, more preferably F68. The Pluronic polyols are commercially available from BASF Wyandotte Corp. (101 Cherry Hill Road, P.O. Box 181, Parsippany, NJ 07054, U.S.A.).

If the protective agent is not also functioning as an emulsifier, the media of this invention further comprise another emulsifier that acts to emulsify the lipid component in conjunction with another emulsifier present at a small concentration in the lipid component itself as described below. Such an emulsifier alternative to a protective agent/emulsifier is preferably a detergent, preferably non-ionic which is non-toxic to the insect cell culture at the concentrations required for emulsification of the lipid component.

The introduction of the lipid component in the form of a microemulsion enhances the availability of lipids in the media to the cells. One option for emulsifying the lipid component is a dual emulsifier system wherein the protective agent is an emulsifier as well as a protective agent and can act in conjunction with an emulsifier or combination of emulsifiers present in the lipid/organic solvent solution making up the lipid component of the media of this invention. Another option for emulsifying the lipid component of the media of this invention is a system wherein the protective agent is not significantly emulsifying but wherein one or more additional emulsifiersd are present in an aqueous solution which is added to the lipid component organic solution and act in conjuction with the emulsifiers present therein to form a microdemulsion.

Preferred emulsifiers added to the lipid component of the media of this invention include phospholipids, preferably lecithin and non-toxi non-ionic polymeric detergents, preferably a polysorbate compound having the formula:



wherein R is a saturated or unsaturated fatty acid having from 16 to 20 carbons, inclusively

wherein t is an integer between 10 and 30, inclusively; and

wherein u is an integer between 10 and 20, inclusively.

Most preferably the non-toxic, non-ionic, polymeric detergent/emulsifier is polyoxyethylene (20) sorbitan monooleate, otherwise known as polysorbate 80. Such a non-toxic, non-ionic polymeric detergent is commercially available as Tween 80 from ICI Americas Inc. (New Murphy Road & Concord Pike, Wilmington, DE 19897, USA). Another polysorbate 80 is commercially available as Durfax 80 from Durkee Industrial Foods Group/SCM Corp. (900 Union Commerce Bldg., Cleveland, Ohio 44115, USA). Other non-toxic, non-ionic, polymeric detergent candidate emulsifiers can be found in editions of McCutcheon's Emulsifiers and Detergents, supra.

Said non-ionic, non-toxic, polymeric detergent/emulsifier, such as, polysorbate 80, is present in the media used in this invention at a concentration from about 5 mg/l to about 75 mg/l, more preferably from about 20 mg/l to about 30 mg/l, and most preferably about 25 mg/l.

A preferred example of a dual emulsifier system of the media of this invention is the combination of a protective agent/emulsifier, preferably a Pluroniic polyol, more preferably Pluronic F68 or Pluronc F88, and still more preferably Pluronic F68, and a non-toxic, non-ionic polymeric detergent, preferably a polysorbate compound, and more preferably polysorbate 80.

The lipid component is preferably added to the bulk of the media of the invention as a microemulsion. An advantage of the lipid component being in the form of a microemulsion, in addition to enhancing the availability of the lipids to the insect cells, is in providing the option of not having to filter sterilize the lipid component and the rest of the media components separately. The lipid component in the form of a microemulsion can be added to the media without being filter sterilized, and the entire media can then be filter sterilized without the concern that lipids, for example, in globular form, could be lost during the filter sterilization process. For large scale production, such an advantage is significant.

An exemplary lipid component of the media used in this invention comprises per liter of media:

10 mg cod liver oil

25 mg Tween 80

41.5 mg cholesterol

2 mg alpha-tocopherol

1 ml ethanol.

To the optionally filtered/sterilzied lipid component solution (1 ml), then, in this exemplification, 10 ml of 10% Pluronic F68 in water (optionally filtered/sterilizsed) is slowly added with agitation as by vortexing.

The peptone component of the serum-free media of this invention can be selected from a wide variety of hydrolyzed protein products, either alone or in combination, including, without limitation, ox liver digest, such as Panmede (commercially available from Paines & Byrnes Ltd., Greenford, England), yeast extract, such as Yeastolate (preferably TC Yeastolate from Difco, USA), caseine digest, such as Bactocasitone (commercially available from Difco, USA), tryptose phosphate broth (TPB) wherein tryptose is the peptone, Lactalbumin Hydrolyzate (LH) (commercially available from Difco, USA) gelatin peptone, glycerin-gelatine peptone, and beef peptone among many other proteolytic digest products of proteins.

Preferably the peptone component is composed of peptone fractions either alone or in combination selected from the group comprising TPB, caseine digest preferably Bactocasitone, ox liver digest preferably Panmede, yeast extract, preferably Yeastolate, and Lactalbumin Hydrolyzate (LH). More preferably, the peptone component comprises either LH or Yeastolate, alone or in combination. Still more preferably, the peptone component comprises either a combination of LH and Yeastolate or Yeastolate alone.

The total peptone concentration present in the media of this invention can be as high as the sum of the highest concentrations of the individual peptone fractions wherein said highest concentration for each peptone fraction is that which is non-toxic and non-inhibitory to cell growth and wherein the total peptone concentration of said highest, non-toxic, non-inhibitory concentrations of the peptone fractions is similary non-toxic and non-inhibitory to cell growth. The highest peptone concentrations can vary not only with the particular peptone fractions used but also with the particular insect cell line that is selected. In general, preferred total peptone concentration present in the media of this invention can range from about 1 g/l to about 12 g/l, more preferably from about 2 g/l to about 8 g/l, and still more preferably from about 3 g/l to about 5 g/l.

Example I

A. Construction of New Baculovirus Transfer Vectors

A.1. Construction of pAcC1

pAcC1 is similar to pAc401 (described previously in Section C.5. except that the recognition site for EcoRI endonuclease has been removed. To accomplish this, pAc401 was digested to completion with EcoRI and the ends were made blunt using Klenow fragment. After ligation and transformation, candidates were screened for the absence of an EcoRI site.

A.2. Construction of pAcC2

pAcC2 is similar to pAc436 (described previously in Section C.5. except that the recognition site for EcoRI endonuclease has been removed. To accomplish this, pAc436 was digested to completion with EcoRI and the ends were made blunt using Klenow fragment. After ligation and transformation, candidates were screened for the absence of an EcoRI site.

A.3. Construction of pAcC3

pAcC3 differs from pAcC2 in that an NcoI restriction site has been introduced at the ATG translational start of the polyhedrin gene. To accomplish this the new transfer vector, pAcC2, was digested to completion with SmaI endonuclease. Following phenol extracton and ethanol precipitation, SmaI digested pAcC2 was dissolved in TE buffer (10 mM Tris-HCl pH 7.4; 1 mM EDTA). In a 50 µl volume of ExoIII buffer (50 mM Tris-HCl pH 8.0; 5 mM $MgCl_2$; 10 mM beta-mercaptoethanol), 10 µg of SmaI digested pAcC2 was treated with 50 units of E. coli Exonuclease III (ExoIII) at 30°C for 5 minutes. The sample was phenol extracted and ethanol precipitated twice. Then 50 pmoles of a primer EK85, 5'AACCTATAAACCATGGCGGCCCGG3', was kinased with cold ATP in a 20 µl reaction volume (50 mM Tris-HCl pH 7.8; 10 mM $MgCl_2$; 10 mM beta-mercaptoethanol). To 5 µg of ExoIII treated pAcC2 was added 10 pmoles of kinased EK85 in a final volume of 20 ml NET (100 mM NaCl; 10 mM Tris-HCl pH 7.5; 1 mM EDTA) buffer. To anneal the plasmid and primer, the reaction was heated to 65°C for 10 minutes, incubated at 37°C for 10 minutes and placed on ice. The extension reaction was performed by adding 20 µl 2 x Klenow buffer (40 mM Tris-HCl pH 7.5; 20 mM MgCl2; 2 mM beta-mercaptoethanol) containing 1 µl 10 mM dNTPs, 1 µl 10 mMATP, 1 µl (about 2 units) Klenow fragment and 1 µl (about 1-2 units) T4 DNA ligase. The reaction was incubated at 16°C for about 4 hours and then transformed into MM294. Minilysates were screened by analyzing for the presence of an NcoI site. Miniprep DNA was then used to retransform and obtain the desired pure clone.

A.4 Construction of pAcC4 and pAcC5

pAcC4 and pAcC5 are derivatives of pAcC3 containing a polylinker sequence at the SmaI site. The polylinker contains recognition sites for restrictionendonucleases SmaI, KpnI, PstI, BglII, XbaI (cleavable when DNA is unmethylated), EcoRI, BamHI and BclI. pAcC4 contains the sequence in one orientation while pAcC5 contains the polylinker in the opposite orientation (see Figure 4). To construct these vectors pAcC3 was digested to completion with XmaI endonuclease and ligated with two complementary self-annealed oligomers having the sequence

5'-CCGGGTACCTGCAGATCTAGAATTCGGATCCTGATCA-3'
3'- CATGGACGTCTAGATCTTAAGCCTAGGACTAGTGGCC-5'

After transformation of MM294, miniprep DNAs of transformants were analyzed for the presence of restriction sites in the polylinker sequence.

Example II

A. Isolation of cDNA Encoding Human CSF-1

A.1 pcCSF-17

The human derived pancreatic carcinoma cell line MIA PaCa-2 was used as a source of mRNA to validate probes and for the formation of a cDNA library containing an intronless form of the human CSF-1 coding sequence. The MIA PaCa cell line produces CSF-1 at a level approximately 10 fold below that of the murine L-929 cells.

Negative control mRNA was prepared from MIA PaCa cells maintained in serum-free medium, i.e. under conditions wherein they do not produce CSF-1. Cells producing CSF-1 were obtained by reinducing CSF-1 production after removal of the serum.

Cells were grown to confluence in roller bottles using Dulbecco's Modified Eagles' Medium (DMEM) containing 10% fetal calf serum, and produced CSF-1 at 2000-6000 units/ml. The cell cultures were washed, and reincubated in serum-free DMEM to suppress CSF-1 formation for negative controls. No detectable CSF-1 was produced

after a day or two. Reinduced cells were obtained by addition of phorbol myristic acetate (100 ng/ml) to obtain production after several days of 1000-2000 units/ml.

The mRNA was isolated by lysis of the cell in isotonic buffer with 0.5% NP-40 in the presence of ribonucleoside vanadyl complex (Berger, S. L., et al. Biochemistry (1979) 18:5143) followed by phenol chloroform extraction, ethanol precipitation, and oligo dT chromatography, and an enriched mRNA preparation obtained. In more detail, cells were washed twice in PBS (phosphate buffered saline) and are resuspended in IHB (140 mM NaCl, 10 mM Tris, 1.5 mM $MgCl_2$, pH 8) containing 10 mM vanadyl adenosine complex (Berger, S. L. et al. supra).

A non-ionic detergent of the ethylene oxide polymer type (NP-40) was added to 0.5% to lyse the cellular, but not nuclear membranes. Nuclei were removed by centrifugation at 1,000 x g for 10 minutes. The postnuclear supernatant was added to two volumes of TE (10 mM Tris, 1 mM ethylenediaminetetraacetic acid (EDTA), pH 7.5) saturated phenol chloroform (1:1) and adjusted to 0.5% sodium dodecyl sulfate (SDS) and 10 mM EDTA. The supernatant was reextracted 4 times and phase separated by centrifugation at 2,000 x g for 10 minutes. The RNA was precipitated by adjusting the sample to 0.25 M NaCl, adding 2 volumes of 100% ethanol and storing at -20°C. The RNA was pelleted at 5,000 x g for 30 minutes, washed with 70% and 100% ethanol, and was then dried. Polyadenylated (poly A^+) messenger RNA (mRNA) was obtained from the total cytoplasmic RNA by chromatography on oligo dT cellulose (Aviv, J., et al., Proc. Natl. Acad. Sci. (1972) 69:1408-1412). The RNA was dissolved in ETS (10 mM Tris, 1 mM EDTA, 0.5% SDS, pH 7.5) at a concentration of 2 mg/ml. This solution was heated to 65°C for 5 minutes, then quickly chilled to 4°C. After bringing the RNA solution to room temperature, it was adjusted to 0.4 M NaCl and was slowly passed through an oligo dT cellulose column previously equilibrated with binding buffer (500 mM NaCl, 10 mM Tris, 1 mM EDTA, pH 7.5 0.05% SDS). The flowthrough was passed over the column twice more. The column was then washed with 10 volumes of binding buffer. Poly A^+ mRNA was eluted with aliquots of ETS,

extracted once with TE-saturated phenol chloroform and was precipitated by the addition of NaCl to 0.2 M and 2 volumes of 100% ethanol. The RNA was reprecipitated twice, was washed once in 70% and then in 100 % ethanol prior to drying.

Total mRNA was subjected to 5-20% by weight sucrose gradient centrifugation in 10 mM Tris HCl, pH 7.4, 1 mM EDTA, and 0.5% SDS using a Beckman SW40 rotor at 20°C and 27,000 rpm for 17 hours. The mRNA fractions were then recovered from the gradient by ethanol precipitation, and injected into Xenopus oocytes in the standard translation assay. The oocyte products of the RNA fractions were assayed in the bone marrow proliferation assay (or in the bone marrow proliferation assays of Moore, R. N., et al., J. Immunol. (1983) 131:2374, and of Prystowsky, M. B., et al., Am. J. Pathol. (1984) 114:149) and the fractions themselves were assayed by dot blot hybridization to a 32-mer probe corresponding to the DNA in the second exon of the genomic sequence (exon II probe). The overlining in Figure 1 shows the exon II probe.

The bone marrow proliferation assay results of the supernatants from the Xenopus oocytes did not correlate exactly with the dot-blot results. The most strongly hybridizing fraction, 11, corresponds to 18S, while the most active fractions 8 and 9 correspond to 14-16S. Fraction 8, 9 and 11 were used to form an enriched cDNA library as described below.

The mRNA was also fractionated on a denaturing formaldehyde gel, transferred to nitrocellulose, and probed with exon II probe. Several distinct species ranging in size from 1.5 kb to 4.5 kb were found, even under stringent hybridization conditions. To eliminate the possibility of multiple genes encoding CSF-1, digests of genomic DNA with various restriction enzymes were subject to Southern blot and probed using pcCSF-17 DNA. The restriction pattern was consistent with the presence on only one gene encoding CSF-1.)

The enriched mRNA pool was prepared by combining the mRNA from the gradient fractions having the highest bone marrow proliferative activity, although their ability to hybridize to probe

is relatively low (14S-16S) with the fractions hybridizing most intensely to probe (18S). Higher molecular weight factions which also hybridized to exon II probe were not included because corresponding mRNA from uninduced MIAPaCa cells also hybridized to exon II probe.

cDNA libraries were prepared from total or enriched human mRNA in two ways. One method uses gt10 phage vectors and is described by Huynh, T. V., et al., in DNA Cloning Techniques: A Practical Approach IRL Press, Oxford 1984, D. Glover, ed.

A preferred method uses oligo dT priming of the poly A tails and AMV reverse transcriptase employing the method of Okayama, H., et al., Mol. Cell Biol. 1(1983) 3:280-289, incorporated herein by reference. This method results in a higher proportion of full length clones than does poly dG tailing and effectively uses as host vector portions of two vectors therein described, and readily obtainable from the authors, pcDV1 and pL1. The resulting vectors contain the insert between vector fragments containing proximal BamHI and XhoI restriction sites; the vector contains the pBR322 origin of replication, and Amp resistance gene and SV40 control elements which result in the ability of the vector to effect expression of the inserted sequences in COS-7 cells.

A 300,000 clone library obtained from above enriched MIAPaCa mRNA by the Okayama and Berg method was then probed under conditions of high stringency, using the exon II probe. Ten colonies hybridizing to the probe were picked and colony purified. These clones were assayed for the presence of CSF-1 encoding sequences by transient expression in COS-7 cells. The cloning vector, which contains the SV40 promoter was used per se in the transformation of COS-7 cells.

Plasmid DNA was purified from the 10 positive clones using a CsCl gradient, and the COS-7 cells transfected using a modification (Wang, A. M., et al., Science (1985) 228:149) of the calcium phosphate coprecipitation technique. After incubation for three days, CSF-1 production was assayed by subjecting the culture supernatants to the radioreceptor assay performed substantially as disclosed by Das, S. K., et al., Blood (1981) 58:630, and to a colony stimulation (bone

marrow proliferation) assay performed substantially as disclosed by Prystowsky, M. B., et al., Am. J. Pathol. (1984) 114:149. Nine of the ten clones picked failed to show transient CSF-1 production in COS-7 cells. One clone, which did show expression, was cultured, the plasmid DNA isolated, and the insert was sequenced. The DNA sequence, along with the deduced amino acid sequence, are shown in Figure 1. The full length cDNA is 1.64 kb and encodes a mature CSF-1 protein of 224 amino acids. The clone was designated CSF-17 with Cetus depository number CMCC 2347 and was deposited with the American Type Culture Collection on June 14, 1985, as accession No. 53149. The plasmid bearing the CSF-1 encoding DNA was designated pcCSF-17.

A.2. Additional Clones

pcCSF-17, prepared as described above, was used as a probe to obtain additional CSF-1 encoding clones from a human cDNA library. Total mRNA was prepared from MIAPaCa cells exactly as described above and used to obtain a cDNA library in gt10 phage vectors by ligating the reverse transcripts to EcoRI linkers and inserting the EcoRI digest of the cDNA thus provided into the EcoRI site of ggt10, as described by Huynh, T. V., et al., in DNA Cloning Techniques: A Practical Approach, IRL Press, Oxford, 1984, D. Glover, ed.

Over one million phage were screened using a single-stranded highly labeled probe derived from CSF-17 using standard procedures, which are briefly summarized as follows.

A BglI-AvaII fragment of pcCSF-17 DNA, which includes the entire coding sequence for CSF-1 plus about 600 bp of 3' untranslated region was inserted into M13, and a labeled second strand synthesized as follows: Approximately 1 pmol of M13 containing the single-stranded BglI-AvaII-digested pcCSF-17 was annealed with 10 pmol of sequencing primer in 20 mM Tris, pH 7.9, 10 mM $MgCl_2$, 100 mM NaCl, and 20 mM beta-mercaptoethanol at 67°C for 5 minutes and then transferred to 42°C for 45 minutes. The annealed preparation was supplied with 100 μmol each of dATP, dCTP, and dTTP, and 2.5 μmol P^{32}-labeled (alpha)GTP, along with 5 U Klenow fragment. The reaction was

incubated at 37° C for 20 minutes, and the DNA recovered on a P-6 dG (Bio-Rad) spin column and boiled for 10 minutes to separate the strands.

The probes, thus prepared, were used to screen the plaques (which had been transferred to nitrocellulose) by hybridization under stringent conditions (50% formamide, 5 x SSC, 5 x Denhardt's) at 42°C for 18 hours.

Approximately 150 phage plaques were positive, and 20 which were particularly strongly hybridized were selected for plaque purification to obtain individual clones.

The 20 clones were then subjected to hybridization under the same conditions to the oligonucleotide GM11, which has the sequence complementary to nucleotides 506-545 in Figure 2. As described above, this sequence is an exact match to that portion of the human genomic sequence which corresponds to the "extra" portion of the murine cDNA, described below, that encodes the "extra" 295 amino acid segment in the "long form" of the murine CSF-1 protein.

The complete DNA sequence for the pertinent coding regions of the cDNA insert in clone 4 along with the deduced amino acid sequence, are shown in Figure 2. The sequence was derived by integrating the known sequence of the genomic clone, phCSF-1a, described above, using the 295 amino acid "extra" insert of the murine sequences described below as a guide to deduce the complete sequence shown. The sequence depicted shows the splicing of the "extra" segment, sufficient to encode 298 "extra" amino acids contained in the gene at the 5' side of exon 6, into the sequence of pcCSF-17 between the first nucleotide of the codon for the Gly residue at amino acid position 150 into reading frame with the remaining CSF-17 sequence. The insert changes the codon at 150 to a codon for aspartic acid, the subsequent codon at the end of the insert is reconstituted to a Gly, and the remaining sequence of residues continuing with His-Glu-Arg etc. down to the C-terminal Val residue remain the same as in pcCSF-17.

Two of the clones, 4 and 25, were cloned into M13mp18 or M13mp19 for sequencing to confirm the results shown in Figure 2 (only clone 4 sequence shown) using the EcoRI restriction sites. These two clones appeared identical from restriction enzyme analysis. They were then subclones into the modified Okayama-Berg vector pcDB, which was prepared from the published Okayama-Berg vectors pcDV1 and pL1 (Okayama, H., et al., Mol. Cell Biol. (1983) 3:280-289) as follows:

pcDV1 was disgested with HindIII and KpnI and the large fragment, providing Amp^r, the pBR322 origin of replication, and a polyadenylation site, was isolated. pL1 was digested with HindIII and PstI and the small fragment, providing the SV40 promoter and origin of replication, was isolated. These fragments were religated in a three-way ligation with the synthetic, KpnI/PstI-digested oligonucleotide fragment

CTGCAGGAGCTCAGATCTTCTAGAGAATTCTCGAGCGGCCGCATCGATGGTACC
GACGTCCTCGAGTCTAGAAGATCTCTTAAGAGCTCGCCGGCGTAGCTACCATGG

to obtain pcDB, a plasmid corresponding to pcD-x shown in the reference, wherein "x" is replaced by a polylinker. Thus, pcDB contains the SV40 early promoter and an immediately downstream linker followed by a polyadenylation site. Sequences ligated into the polylinker region should be expressed under the control of the SV40 early promoter.

Before testing expression, because clones 4 and 25 appeared to be missing some 5' end sequences as compared to CSF-17, the upstream portions from pcCSF-17 were substituted for those of clones 4 and 25.

The protocol for this substitution was as follows: pcCSF-17 was digested with SmaI, which cuts at the extreme 5' end of the untranslated sequence (see Figure 1) and the linearized plasmid was ligated to EcoRI linkers and resealed. The religated plasmid was then digested with EcoRI, which removes the entire coding region from just upstream of the SmaI site at the extreme 5' end to the EcoRI site immediately downstream of the stop codon. This was ligated into the polylinker of pcDB at the EcoRI site. The coding sequences downstream

of the BstXI site, which site is located at approximately the codon for amino acid 8 of the CSF-17 mature protein sequence (see Figure 1) was removed by digestion with BstXI and KpnI. (KpnI cuts into the linker slightly downstream of the EcoRI site past the stop codon.) This deleted downstream sequence was replaced by the analogous BstI/KpnI fragment from mp13-4 and mp13-25. The resulting clones, pcDBhuCSF-4 and pcDBhuCSF-25 thus contained the coding sequences downstream of the codon for approximately amino acid 8 from clones 4 and 25, respectively, and the upstream sequences from pCSF-17. The ligated sequences are in reading frame and under control of the SV40 early promoter.

B. Construction of Recombinant Baculovirus Transfer Vectors Containing the CSF-1 Gene

B.1. Construction of pAcM1 and pAcM2

pAcM1 was constructed by taking advantage of the SmaI and EcoRI sites located outside the CSF-1 coding sequence but within the insert containing the CSF-1 gene in pcCSF-17 (see Kawasaki, E. S. et al., (1985) supra; SmaI recognition site begins at bp 13 and EcoRI begins at bp 953). The pAc611 transfer vector was doubly digested with EcoRI and SmaI endonucleases. The 937 bp SmaI-EcoRI fragment from pcCSF-17 containing the CSF-1 coding sequence was isolated by gel electrophoresis and ligated to SmaI-EcoRI digested pAc611 using T4 DNA ligase. E. coli MM294 was transformed with the ligation mixture and transformants were screened for the presence of the 937 bp SmaI-EcoRI CSF-1 fragment.

pAcM2 was constructed in a manner analogous to that described for pAcM1 except that the SmaI-EcoRI fragment from pCSF-BamBcl described in commonly owned PCT Publication No. WO86/04607, supra was inserted into pAc611 rather than the SmaI-EcoRI fragment from pcCSF-17. pCSF-BamBcl differs from pcCSF-17 in that a mutation has been introduced to change the serine at position 159 in the CSF-1 coding sequence to a stop codon. This mutation was accomplished by excising the coding sequence from pcCSF-17 and ligating into M13 for site-specific mutagenesis using the primer,

5'-GAGGGATCCTGATCACCGCAGCTCC-3'.

This results in a new BclI site at codons 159-160. The mutated DNA was excised with BstXI and EcoRI and ligated into BstXI-EcoRI digested pcCSF-17. The ligation mixture was transformed into E. coli DG105, a dam⁻host, and the plasmid DNA isolated. This host was used because BclI is sensitive to dam methylation. The resulting plasmid, pCSF-BamBcl, was the source of the CSF-1 sequence present in pAcM2.

B.2. Construction of pAcM3

pAcM3 was constructed by using M13 site-specific oligonucleotide directed mutagenesis of the pAcM2 vector to delete the CSF-1 5' untranslated leader sequence and to reconstruct the AcNPV polyhedrin gene 5' untranslated leader sequence. The approximately 900 bp EcoRV-EcoRI fragment from pAcM2 was ligated into SmaI-EcoRI digested M13RF. The EcoRV site is located at -96 bp in the polyhedrin 5' leader (see Smith, G. E. et al., Mol. Cell. Biol. 3:2158) and the EcoRI site begins at bp953 following the CSF-1 coding sequence as previously described in Section B1. Using the primer,

5'-GTTTTGTAATAAAAAAACCTATAAATAATGACCGCGCCGGGC-3'

the polyhedrin 5' untranslated leader is juxtaposed with the ATG translational start of CSF-1. The CSF-1 5' untranslated leader sequence as well as the polylinker sequence of the transfer vector are deleted. The mutated DNA sequence (EK82RF) was restricted with XbaI, the ends were made blunt by Klenow repair and a second endonuclease digestion with EcoRI resulted in a XbaI(blunt)-EcoRI fragment which was inserted into EcoRV-EcoRI digested pAc611. After ligation and transformation into DH5, transformants were screened by restriction analysis. The desired construct designated pAcM3 contains a small insertion in the polyhedrin promoter region at the EcoRV site as a result of subcloning the M13 fragment back into pAc611.

B.3. Construction of pAcM4

pAcM4 is an in frame translational fusion vector constructed by cloning a mutated CSF-1 cDNA fragment into pAcC1. The source of

the CSF-1 cDNA fragment was pCSF-gly150 described in PCT Publication No. WO86/04607, supra which contains a TGA stop codon instead of histidine codon at position 151. It was prepared from the pcCSF-17 insert by site-specific mutagenesis using the appropriate primer,

5'-AGCCAAGGCTGATCAAGGCAGTCC-3'.

Thus, the downstream portion of the coding sequence was excised from pcCSF-17 with BstXI-EcoRI, put into an M13 vector for mutagenesis and returned after plaque purification as a BstXI-EcoRI insert. The resulting plasmid is designated pCSF-gly150.

The SmaI-EcoRI fragment (bp 13-953) in pCSF-gly150 was then inserted into an M13 vector for a second round of mutagenesis. The objective this time was to introduce a SmaI recognition site starting 3 bp after the ATG translational start codon of the CSF-gly150 coding sequence. This was accomplished by site-specific mutagenesis using the appropriate primer 5'-GCCCGTATGTCCCCGGGGGGCGCCG-3'. The mutagenized DNA in M13 is designated EK83RF.

EK83RF was used as the source of the CSF mutein used in the construction of pAcM4. EK83RF was digested with EcoRI endonuclease, repaired with Klenow fragment and digested with SmaI endonuclease. The 770 bp fragment containing the CSF sequence was gel purified and ligated into SmaI digested pAcC1, using T4 DNA ligase. The ligation mixture was used to transform MM294 and transformants were screened by minilysates or colony hybridization.

The desired construct pAcM4 thus contains the intact polyhedrin gene promoter and 5' untranslated leader but three altered codons in the CSF-1 signal peptide. Instead of Met-Thr-Ala-Pro-Gly...the sequence in this construct encodes Met-Arg-Pro-Gly-Gly... The CSF-1 5' untranslated leader sequence has been removed so that all of the sequence 5' to the ATG translational start is derived from the AcNPV polyhedrin gene (see Figure 5).

B.4 Construction of pAcM5 and pAcM6 Containing "Long Form" CSF-1

pAcM5 was prepared by placing the coding region of CSF-4 from pcDBhuCSF-4 into pAc610. pcDBhuCSF-4 was digested with EcoRI

and the 1826 bp fragment containing the 522 amino acid coding sequence of the "long form" of CSF-1 was isolated by gel electrophoresis. The pAc610 transfer vector was also digested with EcoRI endonuclease and ligated with the EcoRI fragment containing the CSF-1 gene. E. coli MM294 were transformed with the ligation mixture and transformants were screened for hybridization to the oligonucleotide. GM11 (described in Section A.2) which hybridizes to the extra amino acid sequence not found in pcCSF-17. The resulting construct was designated pAcM5.

pAcM6 was constructed by excising the 250 bp StuI (positions 511-821 bp on the pcCSF-17 sequence) fragment from pAcM4 (described in Section B.3) and replacing it with the 1141 bp StuI (positions 343-1484 bp on the CSF-4 sequence) fragment from pcDBhuCSF-4. The 1141 bp StuI fragment from pcDBhuCSF-4 contains the sequence from within amino acid 101 to within amino acid 184 of the shorter pcCSF-17 CSF sequence, with an additional coding sequence for 298 amino acids inserted after amino acid 149. Said in another way, the resulting construction, pAcM6, has the same 5' and 3' sequence as found in pAcM4 with the additional 894 bp of additional coding sequence from pcDB huCSF-4 inserted within the CSF-1 coding sequence.

B.5. Construction of pAcM9

pAcM9 was constructed by replacing a fragment of pAcM4 with a mutated fragment of CSF-1 cDNA derived from M13 clone EK90/91. The mutations in this fragment alter the coding sequence of pAcM4 such that amino acids Asn122 and Asn140 are replaced with Gln.

EK90/91 was constructed as follows. The 983 bp SmaI-EcoRI fragment from pCSF-17-asp59-gly150 was cloned into M13mp18 and subjected to oligonucleotide directed mutagenesis using the primer 5'-GAATGTCTTCCAAGAAACAAAGA-3' to produce M13 clone EK90 (codon 122 altered to Gln). EK90 was then mutated with the primer 5'-CAAGAACTGTCAAAACAGCTTTG-3' to produce the double mutant EK90/91 (codon 122 and 140 altered to Gln). The mutated 940 bp SmaI-EcoRI fragment from EK90/91 was then subcloned into pUC18 to product pUC90/91.

The construction pAcM9 was completed by excising the 250bp StuI fragment (511-821bp in pCSF17) from pACM4 and ligating it to the 250bp from pUC90/91. The ligated DNA was transformed into MM294 and the pAcM9 clone was identified by colony hybridization.

B.6. Construction of pAcM10

pAcM10 was constructed by inserting a CSF-1 sequence modified by the introduction of an NcoI site at the ATG translational start into the pAcC4 transfer vector. The modified CSF-1 sequence was derived as follows. The double mutein, pCSF-17-asp59-gly150, described in PCT Publication No. WO86/04607, supra was constructed by a first site specific mutagenesis to change amino acid 59 to aspartic acid and create a new EcoRV site at codons 59-60 using the primer 5'-GGTACAAGATATCATGGAG-3'. The resulting plasmid, pCSF-17-asp59, was digested with BstXI and EcoRI to place the C-terminal fragment into M13 for a second site-specific mutagenesis with the same primer described in Section B.3 for the generation of pCSF-17-gly150. The mutated fragment was then returned to the vector to obtain pCSF-17-asp59-gly150.

pCSF-17-asp59-gly150 was digested with XmaI (bp 13-18 in pcCSF-17) and NarI (bp 47-52, bp 99-104, bp 191-196 in pcCSF-17) to completion. NarI cut twice within the 5' untranslated sequence of CSF-1 and the large NarI-XmaI fragment containing the CSF-1-asp59-gly150 sequence from amino acid 6 on through the vector was purified by gel electrophoresis. Complementary primers having the sequence

5'-CCGGGACCATGGCCGCGCCGGG-3'
3'-CTGGTACCGGCGCGGCCCGC-5'

were self annealed and ligated to the NarI-XmaI fragment containing CSF-1 sequence. Transformants of E. coli MM294 were screened for the presence of the NcoI site introduced in the primer sequence.

pAcM10 was constructed by digesting pCSF-17-NcoI with NcoI and BamHI. pAcC4 was also doubly digested with NcoI and BamHI and after fragment isolation, ligation and transformation, minilysates of E. coli DH5 condidates were screened for the appropriate NcoI-BamHI fragment sizes.

B.7. Construction of pAcM11

pAcM11 was produced by replacing a fragment of pAcM6 with a mutated fragment of CSF-1 cDNA derived from pUC90/91 (described above). The mutations in this fragment alter the 522 amino acid coding sequence of pAcM6 such that amio acids Asn122 and Asn140 are replaced with Gln. pUC90/91 and pcDBhuCSF4 (described in Section A.2) were cleaved with both EcoRI and BsmI. The 1137 bp BsmI (position 477) to EcoRI (position 1614) fragment from pcDBhuCSF4 was agarose-gel purified and ligated to the large fragment of pUC90/91. The ligation reaction was transformed into MM294 cells and colonies were screened by hybridization to the oligomer GM11 (described in Section A.2). The resultant plasmid, pUC90/91EB, contains the entire coding region of CSF4 with the changes previously mentioned. The 1138bp StuI fragment from pUC90/91EB (343-1481bp) was exchanged with the corresponding fragment in pAcM6 to produce pAcM11. pAcM11 was transformed into MM294 and isolated by colony hybridization.

B.8. Construction of pAcM12 and pAcM13

pAcM12 and pAcM13 were derived from pAcM6 and pAcM11 respectively by replacing a 721 bp BamHI fragment (between positions 683 and 1404) with the corresponding fragment derived from M13 clone 4/29/33, an m13mp18 mutant of CSF4. The 4/29/33 fragment has a stop codon immediately following the codon for the proline at amino acid position 221. Therefore, pAcM12 encodes a "long form" CSF-1 protein that is truncated after proline 221 "long form" CSF-1 protein that is truncated after proline 221 that may be glycosylated at Asn 122 and Asn 140. pAcM13 encodes a truncated "long form" CSF-1 protein that cannot be glycosylated at those positions. Clone 4/29/33 was produced by oligonucleotide directed mutagenesis of 4/29 with the primer GM33 which changed the Asn122 and Asn140 to Gln residues.

C. Expression of CSF-1 in Insect Cells

Transfection of insect cells, plaque purification of recombinant virus and infection of insect cells (10^5cells/ml) with the transfer vectors containing CSF-1 described hereinabove, yielded

between 7500 and 500,000 U/ml of CSF-1 activity in small scale infections of 2-25 ml. Expression levels for each of the vectors are compared in Figure 8.

Protein products obtained from the Baculovirus expression system were analyzed by immunoprecipitation of supernatants from infected Sf9 cells. Samples were eluted from rabbit anti-CV1-CSF-1 covalently coupled to Sepharose beads, run on SDS-PAGE and stained with Commasie blue. Figure 6 shows CSF-1 produced by cells cotransfected with pAcM4 (lane 3), pAcM3(lane 4 and pAcM6 (lane 5).

Since the expression products in most cases were precipitated from cell supernatants it is clear that the products were secreted. Molecular weights of the CSF proteins produced indicated that the signal peptide was cleaved though the precise site of cleavage has not yet been determined. The products also appear to be glycosylated as indicated by their increased molecular weight as compared to the CSF produced intracellularly (in the absence of a signal peptide) in E. coli.

Example III

Isolation and Expression of cDNA Encoding Human G-CSF

A cDNA clone encoding human granulocyte stimulating factor was isolated from the MIA PaCa-2 cell line, and was expressed using a recombinant Baculovirus vector in insect cells. The MIA PaCa-2 cell line described in Yunis, A. A. et al., Experimental Hematol., 12:838-843 (1984), is an established cell line publicly available from the Cell Repository Lines (CRL) collection of the American Type Culture Collection, 12301 Parklawn Avenue, Bethesda, MD 20895 under accession number ATCC CRL 1420. The cDNA clone was sequenced and the corresponding amino acid sequence was deduced.

A. Isolation of G-CSF mRNA From MIA PaCa-2 Cells

Confluent MIA PaCa-2 cells were stimulated in serum free Dulbecco's minimum essential medium (DMEM) for 4 days with phorbol

myristate acetate (50 ng/ml) and retinoic acid (10 μM). The RNA was prepared as described by Chirguin et al.; briefly, the cells were lysed in 5 M guanidine isothiocyanate followed by centrifugation through a 5.7 M cesium chloride (CsCl) cushion; poly A^+ RNA was prepared from the lysed cells by one selection cycle on oligo (dT) cellulose as described in Maniatis et al., supra.

In more detail, cells were lysed in a solution which contained 5 M guanidine isothiocyanate, 0.025 M Na-citrate, pH 7, 0.5% sarcosine and 8% beta-mercaptoethanol. Molecular biology grade CsCl was made up to 5.7 M or 40% w/v and buffered with 0.02 M Tris pH 7.5 and 0.002 M Na-EDTA. All solutions were prepared under RNase free conditions and passed through 0.45 Millipore filters before use. The lysed cells were then layered onto SW28 ultracentrifuge tubes which contained layers of 10 ml 5.7 M CsCl and 6 ml 40% CsCl.

After centrifugation at 26,000 rpm for 18 hours, the RNA pellet was dissolved in H_2O and ethanol precipitated twice. Polyadenylated (Poly A^+) messenger RNA (mRNA) was obtained by chromatography of the total RNA on oligo (dT) cellulose as described in Maniatis, supra (at page 197).

The RNA was dissolved in sterile H_2O and heated to 65°C for five minutes. An equal volume of a solution containing 0.040 M Tris Cl pH 7.6, 1.0 M NaCl, 0.002 M EDTA and 0.2% SDS was quickly added and the sample was cooled to room temperature. The sample was then loaded on an oligo (dT) column that had been equilibrated with a buffer containing 0.020 M Tris pH 7.6, 0.5 M NaCl, 0.001 M EDTA and 0.1% SDS. The flow through was collected, heated to 65°C, cooled to room temperature and passed over the column once more. The column was then washed with 10 volumes of wash buffer (0.02 Tris, 0.1 M NaCl, 0.001 M EDTA, 0.1% SDS). Poly A^+ mRNA was eluted with aliquots of 0.01 M Tris pH 7.5, 0.001 M EDTA and ethanol-precipitated twice.

334 μg of MIA PaCa-2 Poly A^+ mRNA was fractionated on 5-25% by weight sucrose gradient centrifugation in 20 mM Tris HCl, pH 7.5, 1 mM EDTA, and 0.5% sarcosine using a Beckman SW40 rotor at 20°C and 27,800 rpm for 16 hours. The mRNA fractions were collected in 400 ~l

fractions and ethanol precipitated twice. Fractions were pooled and resuspended in 15 µl H_2O.

Northern blots were prepared by the electrophoresis of 5 µg of poly A^+ RNA per lane or 1 µl of each pooled RNA fraction through 1% agarose gels containing 0.5 M formaldehyde followed by blotting onto nitrocellulose filters. After baking the filters for 1.5 hours at 80° C, they were prehybridized (5 x SSC, 10 x Denhardt's solution (0.2% polyvinylpyrrolidone, 0.2% Ficoll, 0.2% BSA), 0.1% SDS, 50 mM sodium phosphate pH 7.0, and 100 µg/ml tRNA) for one hour at 55°C. The blots were hybridized for 16 hours at 55°C in a similar solution that also contained 10% dextran sulfate and 10^6 cpm per ml of an oligonucleotide probe labeled with gamma ^{32}P-ATP and polynucleotide kinase. The blot shown in Figure 3B was washed at 55°C in 3 x SSC, 0.1% SDS. The oligonucleotide probe had the sequence 5'-GTAGGTGGCACACAGCTTCTCCTG-3' and was designed based on the sequence of the CHU-2 cDNA clone described by Nagata et al., Nature, 319:415-418 (1986).

The RNA pools that were probe-positive in the above assay were translated in Xenopus laevis oocytes by the injection of 50 nl of RNA into each oocyte as described in Gurdon et al., Nature, 233:177-180 (1971). Supernatants of 10 µl per oocyte were collected after 40 hours and assayed for CSF activity.

The Xenopus laevis supernatants from each hybridization fraction were assayed for CSF activity in a murine bone marrow cell proliferation assay as described in Moore, et al., J. Immunol., 131:2374-2378 (1983). Briefly, in this assay, 5 x 10^4 murine bone marrow cells per/well were incubated in 96-well plates (12 x 8) with serially diluted Xenopus laevis oocyte supernatants made from positively hybridizing mRNA fractions. After three days, ^{3}H thymidine (0.5 µCi/well) was added, and after six hours the cells were harvested and counted in a liquid scintillation counter.

Peak bone marrow proliferation was found in Xenopus laevis oocyte supernatants made with mRNA fractions that were most strongly positive in Northern blots with the above-described oligonucleotide probe.

B. Identification of G-CSF Clones in a MIA PaCa-2 cDNA Library

A cDNA library was prepared from the enriched MIA PaCa-2 mRNA as described in Kawasaki, et al., Science, 30:291-296 (1985). Briefly, the method used oligo (dT) priming of the poly A^+ tails and AMV reverse transcriptase employing the method of Okayama, H. et al., Mol. Cell Biol., 3:280-289 (1983), which is incorporated herein by reference. This method results in a higher proportion of full length clones than does poly (dG) tailing and effectively uses as host vector portions of two vectors therein described, and readily obtainable from the authors, pcDV1 and pL1. The resulting vectors contain the insert between vector fragments containing proximal BamHI and XhoI restriction sites; the vector contains the pBR322 origin of replication, and ampicillin resistance gene and SV40 control elements which result in the ability of the vector to effect expression of the inserted sequences in COS-7 cells.

A 1.2 x 10^6 clone library in E. coli obtained from the above enriched MIA PaCa-2 mRNA by the Okayama and Berg method was then probed using the same oligonucleotide probe that yielded a positive signal on the most active pooled MIA PaCa-2 mRNA fractions. To probe the library, E. coli containing the Okayama-Berg vectors were grown up on nutrient medium. Colonies were lifted onto nitrocellulose filter papers and were lysed. DNA was fixed to the filter by treatment for five minutes with 0.5 mM NaOH, 1.5 M NaCl. Filters were then washed twice for five minutes each time with 1.5 M Tris pH 8, 3 M NaCl and were air dried and baked at 80°C for two hours.

The filters for the screening were prehybridized and hybridized to the gamma ^{32}P labeled probe as described above in Section B of the example, but both prehybridization and hybridization were carried out at 50°C. Plasmids pP12 and pP28 were determined to be probe positive and were further characterized.

C. Sequencing of G-CSF MIA PaCa-2 Plasmids cDNA

pP12 was digested with BamHI and subcloned into a M13mp19 vector and sequenced using the dideoxy chain-termination method.

The DNA sequence and predicted protein sequence of pP12 are shown in Figure 4. The cDNA insert in pP12 is 1510 base pairs long excluding the poly (dG) and poly (dA) tails; it contains 11 more bases of 5' untranslated sequence than the CHU-2 G-CSF clone. The major difference between this clone derived from MIA PaCa-2 and CHU-2 cDNA clones of Nagata et al. is a 9 base pair insertion (GTGATGGAG) in the CHU-2 clone that would encode the amino acid residues Val-Ser-Glu just prior to cys-36 in the MIA PaCa-2 G-CSF as indicated by the arrow in Figure 4. There are two other differences; an A at position 588 in the MIA PaCa-2 clone (G in the CHU-2 clone) is a silent third base change, and a T at position 1237 in the MIA PaCa-2 clone (C in the CHU-2 clone) is in the 3' untranslated region.

2. Construction of Recombinant Baculovirus Transfer Vectors Containing the G-CSF Gene

D.1. Construction of pAcG1 (also known as pJD2)

The Baculovirus G-CSF expression vector, pAcG1, was constructed using the pAc610 transfer vector and the G-CSF coding sequence from pP12. pAc610 was digested with EcoRI endonuclease and the sticky ends were made blunt using the Klenow fragment as described. A second digestion with BamHI endonuclease prepared the transfer vector for receipt of the G-CSF coding sequence immediately downstream from the polyhedrin gene 5' leader sequence. pP12 containing the G-CSF coding sequence was digested with NcoI endonuclease which contains within its recognition sequence the ATG translational initiation codon of the G-CSF gene. Following Klenow repair to generate blunt ends, pP12 was digested with BamHI endonuclease. The NcoI (blunt)-BamHI fragment containing the G-CSF gene was purified by gel electrophresis. After ligation with T4 ligase and transformation into E. coli MM294 candidates were screened by restriction analysis. Double digestion with EcoRV and BamHI endonucleases resulted in two fragments 1.5 and 8 kb in length.

D.2 Construction of pAcG2

The pAcG1 plasmid lacks the native G-CSF leader sequence but does contain a G-C rich segment from the pAc610 polylinker adjacent to the first ATG of the coding sequence. In addition, this construction has a T at the -3 position from the ATG translational start codon which has been reported to result in low translational efficiency (Kozak, M. Cell, (1986) 44:283). The new transfer vector pAcC3 was designed to eliminate the G-C rich region, restore the complete polyhedrin leader and place an A residue at the -3 position to the translational start.

pAcG2 was constructed by introducing an NcoI-SspI fragment from pP12 containing the G-CSF coding sequence into pAcC3. pP12 was digested with SspI endonuclease which cuts some 40 bp 3' to the translational termination codon of G-CSF to produce blunt ends. A second digestion with NcoI endonuclease results in a 654 bp fragment containing the G-CSF coding sequence (minus leader) which was gel purified. pAcC3 was doubly digested with NcoI and SmaI endonucleases and purified by gel electrophoresis for ligation to the G-CSF fragment. After ligation with T4 ligase and transformation into MM294(?), transformants were screened for presence of insert by digesting with restriction endonucleases. The presence of an NcoI restriction endonuclease recognition site ensured that the translational initiation codon had been regenerated.

E. Expression of G-CSF in Insect Cells

Transfection of insect cells, plaque purification of recombinant virus and infection of insect cells (10^5 cells/ml) with pAcG1 yielded about 90,000 units/ml of G-CSF. Expression levels obtained with pAcG2 after similar procedures were about 2.4×10^6 units/ml of G-CSF. These levels are compared to levels of CSF-1 produced in at least two different constructions in Figure 8.

Protein products obtained from the Baculovirus expression system were analyzed by immunoblotting of supernatants from the infected Sf9 cells. Figure 7 shows the results of this experiment.

Other modifications of the above described embodiments of the invention which are obvious to those of skill in the area of molecular biology and related disciplines are intended to be within the scope of the claims that follow.

Deposits

The materials listed below were deposited with the American Type Culture Collection, Rockville, MD, USA (ATCC). The deposits were made under the provisions of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure and the Regulations thereunder (Budapest Treaty). Maintenance of a viable culture is assured for 30 years from date of deposit. The organism will be made available by ATCC under the terms of the Budapest Treaty, and subject to an agreement between Applicants and ATCC which assures unrestricted availability upon issuance of the pertinent U.S. patent. Availability of the deposited strain is not to be construed as a license to practice the invention in contravention of the rights granted under the authority of any government in accordance with its patent laws.

Recombinant Transfer Vector	CMCC#	ATCC#	Deposit Date
pAcM3			6/12/87
pAcM4			6/12/87
pAcM6			6/12/87
pAcM10			6/12/87
pAcG2			

WHAT IS CLAIMED IS:

1. A recombinant baculovirus transfer vector capable of introducing a colony-stimulating factor (CSF) gene or portion thereof into a baculovirus genome said transfer vector comprising a portion of the baculovirus genome, a bacterial replication origin and a DNA sequence encoding a CSF under the transcriptional control of a baculovirus promoter.

2. A recombinant baculovirus transfer vector according to claim 1 wherein said baculovirus promoter is the polyhedrin gene promoter.

3. A recombinant baculovirus transfer vector according to claim 2 wherein said DNA sequence encoding CSF is translationally fused with at least two codons of the polyhedrin gene wherein at least one of said codons is an initiation codon.

4. A recombinant baculovirus transfer vector according to claims 1, 2 or 3 wherein said DNA sequence encoding a CSF is selected from the group of plasmids consisting of pcCSF-17, pcDBhuCSF-4 or pP12.

5. A recombinant baculovirus transfer vector according to claim 4 wherein said DNA sequence encoding CSF-1 from pcCSF-17 contains a translational stop codon replacing either histidine 151 or serine 159.

6. A recombinant baculovirus transfer vector according to claim 5 wherein a polylinker sequence and 5' untranslated leader sequence of CSF-1 are deleted thereby bringing the polyhedrin gene promoter into juxtaposition with the CSF-1 translational initiation codon.

7. A recombinant baculovirus transfer vector according to claim 5 wherein the codons for the second, third and fourth amino acids differ from those of CSF-1.

8. A recombinant baculovirus transfer vector according to claim 3 wherein a NcoI restriction endonuclease recognition site has been introduced at the ATG translational initiation codon of the polyhedrin gene.

9. A recombinant baculovirus transfer vector according to claim 4 wherein the DNA sequence encoding the native polypeptide leader sequence of G-CSF has been deleted from the DNA sequence encoding G-CSF from pP12.

10. A recombinant baculovirus expression vector for the production of biologically active colony-stimulating factors (CSFs) said expression vector comprising a recombinant baculovirus genome and a DNA sequence encoding a CSF under the transcriptional control of a baculovirus promoter.

11. A method for producing a polypeptide with colony stimulating factor (CSF) activity comprising infecting a susceptible host insect cell with a recombinant baculovirus expression vector wherein said expression vector is a recombinant baculovirus genome comprising at least one DNA sequence encoding a CSF under the transcriptional control of a baculovirus promoter, growing said infected insect cells under suitable conditions and recovering said polypeptide from the culture medium.

12. The method of claim 11 wherein said host insect cells are Spodoptera frugiperda cells.

13. The method of claim 11 wherein said baculovirus genome is derived from Autographa californica, Trichoplusia ni, Rachiplusia ou or Galleria mellonella.

14. The method of claim 11 wherein the recombinant baculovirus transfer vector containing a gene encoding a CSF is selected from the group consisting of pAcM1, pAcM2, pAcM3, pAcM4, pAcM5, pAcM6, pAcM9, pAcM10, pAcM11, pAcM12, pAcM13, pAcG1 or pAcG2.

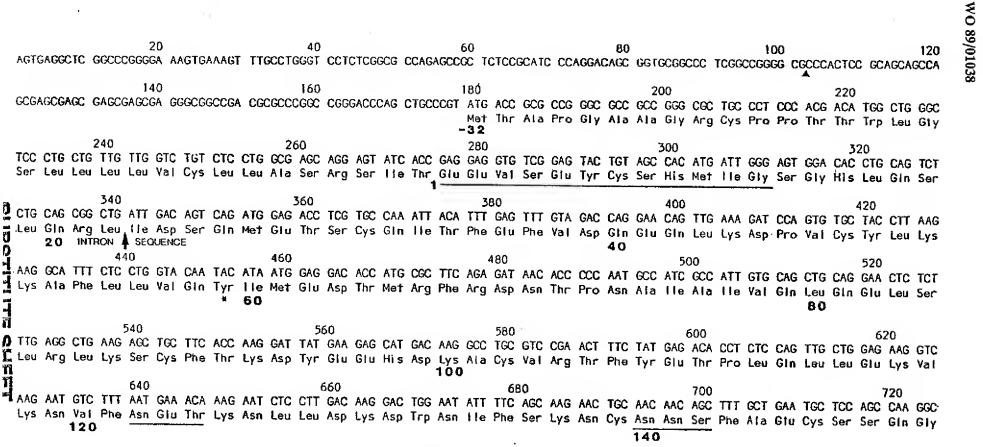


FIG. 1-1

```
                        740                            760                            780                             800                            820
CAT GAG AGG CAG TCC GAG GGA TCC TCC AGC CCG CAG CTC CAG GAG TCT GTC TTC CAC CTG CTG GTG CCC AGT GTC ATC CTG GTC TTG CTG GCC GTC GGA
His Glu Arg Gln Ser Glu Gly Ser Ser Ser Pro Gln Leu Gln Glu Ser Val Phe His Leu Leu Val Pro Ser Val Ile Leu Val Leu Leu Ala Val Gly
                                    160                                                                                       180
                         840                            860                            880                            900                            920
GGC CTC TTG TTC TAC AGG TGG AGG CGG CGG AGC CAT CAA GAG CCT CAG AGA GCG GAT TCT CCC TTG GAG CAA CCA GAG GGC AGC CCC CTG ACT CAG GAT
Gly Leu Leu Phe Tyr Arg Trp Arg Arg Arg Ser His Gln Glu Pro Gln Arg Ala Asp Ser Pro Leu Glu Gln Pro Glu Gly Ser Pro Leu Thr Gln Asp
                                                                   200
                          940                        960                    980                    1000                   1020
GAC AGA CAG GTG GAA CTG CCA GTG TAG AGGGAATTCTA  AGACCCCTCA  CCATCCTGGA  CACACTCGTT  TGTCAATGTC  CCTCTGAAAA  TGTGACGCCC  AGCCCCGGAC
Asp Arg Gln Val Glu Leu Pro Val
            220
      1040                   1060                   1080                   1100                   1120                   1140
ACAGTACTCC  AGATGTTGTC  TGACCAGCTC  AGAGAGAGTA  CAGTGGGACT  GTTACCTTCC  TTGATATGGA  CAGTATTCTT  CTATTTGTGC  AGATTAAGAT  TGCATTAGTT  TTTTTCTTAA

      1160                   1180                   1200                   1220                   1240                   1260
CAACTGCATC  ATACTGTTGT  CATATGTTGA  GCCTGTGGTC  TATTAAAACC  CCTAGTTCCA  TTTCCCATAA  ACTTCTGTCA  AGCCAGACCA  TCTCTACCCT  GTACTTGGAC  AACTTAACTT
                                                                              ▲
      1280                   1300                   1320                   1340                   1360                   1380
TTTTAACCAA  AGTGCAGTTT  ATGTTCACCT  TTGTTAAAGC  CACCTTGTGG  TTTCTGCCCA  TCACCTGAAC  CTACTGAAGT  TGTGTGAAAT  CCTAATTCTG  TCATCTCCGT  AGCCCTCCCA

      1400                   1420                   1440                   1460                   1480                   1500
GTTGTGCCTC  CTGCACATTG  ATGAGTGCCT  GCTGTTGTCT  TTGCCCATGT  TGTTGATGTA  GCTGTGACCC  TATTGTTCCT  CACCCCTGCC  CCCCGCCAAC  CCCAGCTGGC  CCACCTCTTC

      1520                   1540                   1560                   1580                   1600                   1620
CCCCTCCCAC  CCAAGCCCAC  AGCCAGCCCA  TCAGGAAGCC  TTCCTGGCTT  CTCCACAACC  TTCTGACTGC  TCTTTTCAGT  CATGCCCCTC  CTGCTCTTTT  GTATTTGGCT  AATAGTATAT

      1640
CAATTTGCAC  TT
```

FIG. 1–2

FIG. 2–1

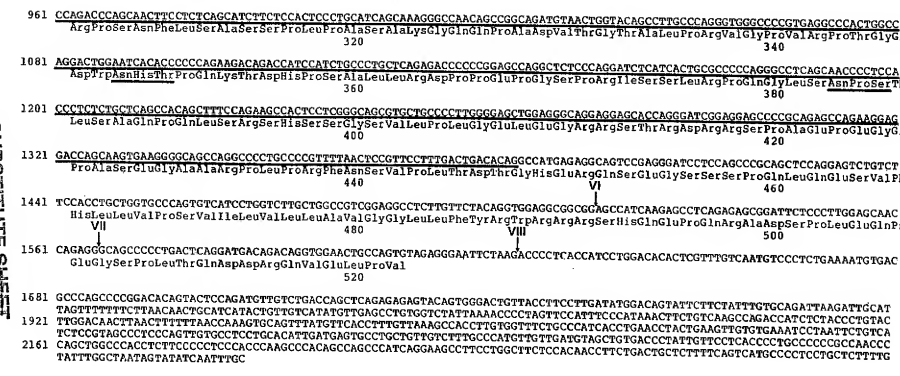


FIG. 2-2

FIG. 3

```
   1 AAAAAACAGCCCGGAGCCTGCAGCCCAGCCCCACCCAGACCCATGGCTGGACCTGCCACC
                                                 MetAlaGlyProAlaThr
                                                 -30

  61 CAGAGCCCCATGAAGCTGATGGCCCTGCAGCTGCTGCTGTGGCACAGTGCACTCTGGACA
     GlnSerProMetLysLeuMetAlaLeuGlnLeuLeuLeuTrpHisSerAlaLeuTrpThr
               -20                                 -10

 121 GTGCAGGAAGCCACCCCCCTGGGCCCTGCCAGCTCCCTGCCCCAGAGCTTCCTGCTCAAG
     ValGlnGluAlaThrProLeuGlyProAlaSerSerLeuProGlnSerPheLeuLeuLys
          -1  1                                  10

 181 TGCTTAGAGCAAGTGAGGAAGATCCAGGGCGATGGCGCAGCGCTCCAGGAGAAGCTGTGT
     CysLeuGluGlnValArgLysIleGlnGlyAspGlyAlaAlaLeuGlnGluLysLeuCys
               20                                  30

 241 GCCACCTACAAGCTGTGCCACCCCGAGGAGCTGGTGCTGCTCGGACACTCTCTGGGCATC
     AlaThrTyrLysLeuCysHisProGluGluLeuValLeuLeuGlyHisSerLeuGlyIle
               40                                  50

 301 CCCTGGGCTCCCCTGAGCAGCTGCCCCAGCCAGGCCCTGCAGCTGGCAGGCTGCTTGAGC
     ProTrpAlaProLeuSerSerCysProSerGlnAlaLeuGlnLeuAlaGlyCysLeuSer
               60                                  70

 361 CAACTCCATAGCGGCCTTTTCCTCTACCAGGGGCTCCTGCAGGCCCTGGAAGGGATCTCC
     GlnLeuHisSerGlyLeuPheLeuTyrGlnGlyLeuLeuGlnAlaLeuGluGlyIleSer
               80                                  90

 421 CCCGAGTTGGGTCCCACCTTGGACACACTGCAGCTGGACGTCGCCGACTTTGCCACCACC
     ProGluLeuGlyProThrLeuAspThrLeuGlnLeuAspValAlaAspPheAlaThrThr
              100                                 110

 481 ATCTGGCAGCAGATGGAAGAACTGGGAATGGCCCCTGCCCTGCAGCCCACCCAGGGTGCC
     IleTrpGlnGlnMetGluGluLeuGlyMetAlaProAlaLeuGlnProThrGlnGlyAla
              120                                 130

 541 ATGCCGGCCTTCGCCTCTGCTTTCCAGCGCCGGGCAGGAGGGGTCCTAGTTGCCTCCCAT
     MetProAlaPheAlaSerAlaPheGlnArgArgAlaGlyGlyValLeuValAlaSerHis
              140                                 150

 601 CTGCAGAGCTTCCTGGAGGTGTCGTACCGCGTTCTACGCCACCTTGCCCAGCCCTGAGCC
     LeuGlnSerPheLeuGluValSerTyrArgValLeuArgHisLeuAlaGlnPro
              160                                 170

 661 AAGCCCTCCCCATCCCATGTATTTATCTCTATTTAATATTTATGTCTATTTAAGCCTCAT
 721 ATTTAAAGACAGGGAAGAGCAGAACGGAGCCCCAGGCCTCTGTGTCCTTCCCTGCATTTC
 781 TGAGTTTCATTCTCCTGCCTGTAGCAGTGAGAAAAAGCTCCTGTCCTCCCATCCCCTGGA
 841 CTGGGAGGTAGATAGGTAAATACCAAGTATTTATTACTATGACTGCTCCCCAGCCCTGGC
 901 TCTGCAATGGGCACTGGGATGAGCCGCTGTGAGCCCCTGGTCCTGAGGGTCCCCACCTGG
 961 GACCCTTGAGAGTATCAGGTCTCCCACGTGGGAGACAAGAAATCCCTGTTTAATATTTAA
1021 ACAGCAGTGTTCCCCATCTGGGTCCTTGCACCCCTCACTCTGGCCTCAGCCGACTGCACA
1081 GCGGCCCCTGCATCCCCTTGGCTGTGAGGCCCCTGGACAAGCAGAGGTGGCCAGAGCTGG
1141 GAGGCATGGCCCTGGGGTCCCACGAATTTGCTGGGGAATCTCGTTTTTCTTCTTAAGACT
1201 TTTGGGACATGGTTTGACTCCCGAACATCACCGACGTGTCTCCTGTTTTTCTGGGTGGCC
1261 TCGGGACACCTGCCCTGCCCCCACGAGGGTCAGGACTGTGACTCTTTTTAGGGCCAGGCA
1321 GGTGCCTGGACATTTGCCTTGCTGGACGGGGACTGGGGATGTGGGAGGGAGCAGACAGGA
1381 GGAATCATGTCAGGCCTGTGTGTGAAAGGAAGCTCCACTGTCACCCTCCACCTCTTCACC
1441 CCCCACTCACCAGTGTCCCCTCCACTGTCACATTGTAACTGAACTTCAGGATAATAAAGT
1501 GTTTGCCTCCAAAAAAAAAAAAAAA
```

FIG. 4

BACULOVIRUS TRANSFER VECTORS

SUBSTITUTE SHEET

FIG. 5

SUBSTITUTE SHEET

FIG. 6

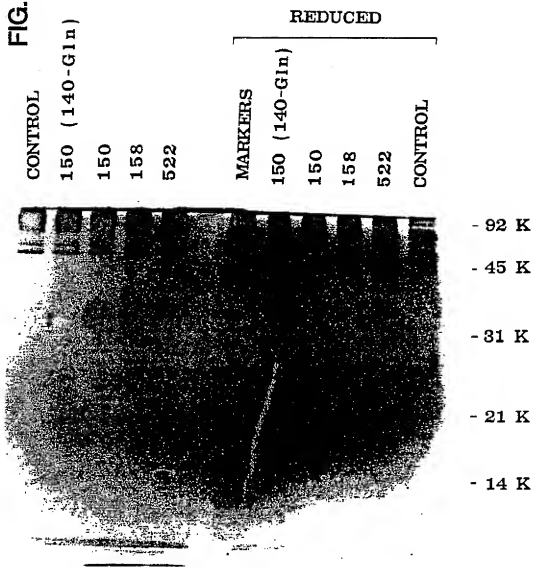

CONTROL
150 (140-Gln)
150
158
522
REDUCED
MARKERS
150 (140-Gln)
150
158
522
CONTROL
- 92 K
- 45 K
- 31 K
- 21 K
- 14 K

FIG. 7

PLASMID	CODING SEQUENCE	CONSTRUCTION OF PLASMID	EXPRESSION (RIA ASSAY) SMALL SCALE INFECTIONS
pAcM1	VAL 224	IN pAc611	7500 U/mL
pAcM2	SER 158	IN pAc611	75,000
pAcM3	SER 158	REMOVE POLYLINKER AND CSF-1 LEADER	320,000
pAcM4	GLY 150	SMA I SITE IN CSF-1, PUT IN pAcC1	500,000 (1,200,000 SCALED-UP)
pAcM5	VAL 522	IN pAc610	24,000
pAcM6	VAL 522	IN pAcM4	100,000
pAcM9	GLY 150, GLN 122, GLN 140	IN pAcM4	24,000
pAcM10	GLY 150	IN pAcC3	500,000
pAcM11	VAL 522, GLN 122, GLN 140	IN pAcM4	13,000
pAcM12	PRO 221	IN pAcM4	>80,000
pAcM13	PRO 221, GLN 122, GLN 140	IN pAcM4	>80,000

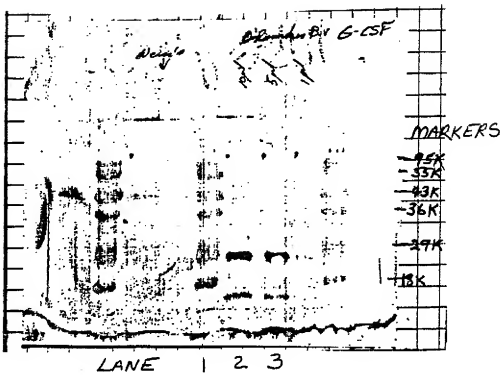


FIG. 8

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US 88/02445

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC[4]: C 12 N 15/00

II. FIELDS SEARCHED

Minimum Documentation Searched [7]	
Classification System	Classification Symbols
IPC[4]	C 12 N

Documentation Searched other than Minimum Documentation to the Extent that such Documents are included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
Y	WO, A, 86/00639 (SANDOZ AG) 30 January 1986 see claims --	1,2,10,11
Y	EP, A, 0127839 (THE TEXAS A&M UNIVERSITY SYSTEM) 12 December 1984 see claims cited in the application --	1,2,10,11
Y	Proc. Natl. Acad. Sci, USA, volume 82, no. 24, December 1985, (Washington DC, US), G.E. Smith et al.: "Modification and secretion of human interleukin 2 produced in insect cells by a baculovirus expression vector", pages 8404-8408 see the whole article --	1,2,10,11
P,X	EP, A, 0272779 (CETUS CORP.) 29 June 1988 see page 10, lines 3-6; claims --	10,11
P,x	Gene, volume 58, nos. 2,3, 1987, Elsevier Science Publishers B.V. (Biomedical ./.	1,2,10,11

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
27th October 1988	28 NOV 1988
International Searching Authority	Signature of Authorized Officer
EUROPEAN PATENT OFFICE	P.C.G. VAN DER PUTTEN

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
	Division), (Amsterdam, NL), A. Miyajima et al.: "Use of the silkworm, Bombyx mori, and an insect baculovirus vector for high-level expression and secretion of biologically active mouse interleukin-3", pages 273-281 see the whole article --	
P,X	Biotechnology, volume 6, January 1988, (New York, US), V.A. Luckow et al.: "Trends in the development of baculovirus expression vectors", pages 47-55 see table 1 --------	1,2,10-13

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. US 8802445 SA 23618

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 15/11/88 The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A- 8600639	30-01-86	AU-A- 4545685	10-02-86
		EP-A- 0188479	30-07-86
		JP-T- 61502682	20-11-86
EP-A- 0127839	12-12-84	AU-A- 2871784	29-11-84
		JP-A- 60037988	27-02-85
		CA-A- 1222213	26-05-87
		US-A- 4745051	17-05-88
EP-A- 0272779	29-06-88	AU-A- 8007087	28-04-88
		WO-A- 8803173	05-05-88

For more details about this annex : see Official Journal of the European Patent Office, No. 12/82